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EFFET DU MAGNÉSIUM ET IMPLICATION DES CANAUX TRPM7 DANS LES FONCTIONS DES CELLULES OSTÉOBLASTIQUES

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TABLE DES MATIÈRES

LISTE DES FIGURES

Chapitre I

Chapitre II

Article 1

Article 2

Article 3

Article 4

Chapitre III

Conclusion

LISTE DES TABLEAUX

LISTE DES ABRÉVIATIONS, DES SIGLES ET DES ACRONYMES

AP-1:	*Activator protein-1*
ATCC:	*American Type Culture Collection*
2-APB:	*2-amino ethoxyphénylborate*
BSA :	*Bas seuil d'activation*
Ca:	*Calcium*
CCDV :	*Canaux calciques voltage-dépendants*
Cdk:	*Kinases cycline-dépendantes*
CRACC:	*Ca^{2+} release-activated Ca^{2+} channel*
ERK:	*Extracellular signal-regulated kinases*
G-2-P:	*Glycérol-2-phosphate*
HC11 :	*Cellules mammaires de souris*
HEK293:	*Human embryonic kidney*
HSA:	*Haut seuil d'activation*
IGF:	*Insulin-like growth factor*
NUDT9:	*Human nucleoside diphosphate linked moiety X-type motif 9*
MAPK:	*Mitogen-activated protein kinases*
M-CSF:	*Macrophage colony-stimulating factor*
Mg^{2+}:	*Magnésium*
MTT :	*Microtiter tetrazolium*
OC :	*Ostéocalcine*
OPG :	*Ostéoprotégérine*
PDGF:	*Platelet-derived growth factor*
PIP_2:	*Phosphatidylinositol* 4,5-bisphosphate
PKC:	*Protein kinase C*

PTH:	Parathormone
RANK-l:	Receptor activator of nuclear factor kappa B ligand
Rb:	Rétinoblastome
RBL :	Leucémiques basophiles
RE :	Réticulum endoplasmique
ROCC:	Receptor-operated Ca^{2+} channel
RGD:	Arginine-glutamine-aspartate
TRP:	Transient receptor potential
TRPC:	Transient receptor potential canonical
TRPM:	Transient receptor potential melastatin
TRPV:	Transient receptor potential vanilloid
TCF:	Ternary complex factor
TGF ß:	Transforming growth factor beta
SMOCC:	Second messenger-operated Ca^{2+} channels
SNARE:	Protéine soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SOC:	Store-operated channel
SOCC:	Store-operated Ca^{2+} channel
STIM1:	Stromal interacting molecule
VAMP2:	Vesicle-associated membrane protein

RÉSUMÉ

Le tissu osseux est en perpétuel renouvellement (processus désigné remodelage osseux) qui se caractérise par la dégradation (résorption) du tissu osseux par les ostéoclastes et la formation d'un nouveau tissu osseux par les ostéoblastes. L'équilibre entre ces deux processus permet le maintien et le renouvellement permanent de la matrice osseuse. Dans bien des cas où l'équilibre est perdu, il y a apparition d'ostéoporose (littéralement : la maladie des os poreux) qui est caractérisée par une masse osseuse réduite due à une dégradation osseuse supérieure à la formation osseuse, une fragilité osseuse et une susceptibilité accrue aux fractures. L'ostéoporose affecte plus de 1,4 million de Canadiens; ainsi 25% des femmes et 13% des hommes de plus de 50 ans souffrent de cette maladie. La réduction de la qualité de vie (diminution de l'estime de soi, réduction ou perte de mobilité et d'autonomie) pour les personnes atteintes d'ostéoporose est énorme. Les coûts pour traiter l'ostéoporose et les fractures qui en résultent sont supérieurs à 1,9 milliard chaque année au Canada. Ces impacts socioéconomiques militent en faveur d'une meilleure compréhension du remodelage osseux. Parmi les facteurs de risque, une diète déficiente en magnésium (Mg^{2+}) a été identifiée comme une condition prédisposant à une réduction graduelle de la masse osseuse et au développement de l'ostéoporose. Des études indiquent qu'une diète réduite en Mg^{2+} entraîne une augmentation du nombre d'ostéoclastes, une diminution du nombre d'ostéoblastes et une perte de la masse osseuse. Les mécanismes assurant l'homéostasie du Mg^{2+} intracellulaire ne sont pas encore parfaitement élucidés. La présente étude vise à déterminer l'importance du Mg^{2+} et l'implication des canaux «melastatin related transient receptor potentiel 7» (TRPM7) au niveau de la prolifération, de la migration et de la différenciation des ostéoblastes ainsi que leur capacité à synthétiser et minéraliser la matrice osseuse. Nos travaux indiquent pour la première fois que les cellules ostéoblastiques expriment les canaux TRPM7 et qu'une réduction du Mg^{2+} extracellulaire est associée à une diminution de l'activité des ostéoblastes (prolifération, migration, différenciation, minéralisation). De plus, nos résultats indiquent que le canal TRPM7 assure l'homéostasie du Mg^{2+} intracellulaire, et que son expression est augmentée dans des conditions de prolifération cellulaire induite par le ''platelet-derived growth factor'' (PDGF), de différenciation et en présence d'un milieu de culture réduit en Mg^{2+}, suggérant son implication dans les fonctions des ostéoblastes. Par ailleurs, une stratégie d'interférence à l'ARN ciblant le TRPM7 des ostéoblastes diminue la prolifération ainsi que la migration basale et induite par le PDGF, en plus de réduire la capacité des ostéoblastes à se différencier et par la suite à minéraliser la matrice osseuse. En conclusion, nos résultats indiquent que les fonctions des ostéoblastes sont favorisées par la présence de concentrations adéquates de Mg^{2+} extracellulaire et des canaux TRPM7. Cette étude souligne l'importance du

Mg^{2+} au niveau des fonctions des cellules ostéoblastiques et nos résultats se veulent en accord avec la diminution de la formation osseuse et le développement de l'ostéoporose associés à une diète déficiente en Mg^{2+}.

Mots clés : TRPM7, ostéoblastes, prolifération, migration, différenciation, PDGF, ostéoporose.

INTRODUCTION

L'ostéoporose est une maladie caractérisée par une faible masse osseuse et une détérioration micro-architecturale du tissu osseux, entraînant une fragilité osseuse et une augmentation du risque de fractures. L'os est en perpétuel renouvellement et grâce à un équilibre entre sa destruction et sa formation, 10 % de notre tissu osseux sont renouvelés chaque année. Ce processus de remodelage est indispensable au maintien des qualités mécaniques et métaboliques de notre squelette. Dans bien des cas où l'équilibre est perdu, il y a apparition d'ostéoporose (littéralement : la maladie des os poreux). Deux acteurs cellulaires sont impliqués dans ce remodelage : les ostéoclastes chargés de la destruction de l'os ancien (résorption), et les ostéoblastes qui agissent en comblant les lacunes de résorption en déposant un nouveau tissu osseux (formation) (Mackie 2003). La fonction principale de l'ostéoblaste est de synthétiser et de minéraliser la matrice osseuse au cours de la croissance du squelette, du renouvellement de la matrice osseuse chez l'adulte et de la réparation osseuse tout au long de la vie. Cette matrice est composée majoritairement de collagène de type I dont le rôle est d'assurer la résistance et l'élasticité de l'os, des propriétés qui sont dépendantes de la quantité et de la qualité du collagène synthétisé.

Parmi les facteurs de risque tels la chute des stéroïdes sexuels à la ménopause et une immobilisation prolongée, une diète faible en Mg^{2+} a été identifiée comme un facteur prédisposant à une réduction graduelle de la masse osseuse et au développement de l'ostéoporose chez l'humain (Rude et Gruber 2004). De plus chez le rat, une diète réduite en Mg^{2+} entraîne une augmentation du nombre d'ostéoclastes, une diminution du nombre d'ostéoblastes et une perte de la masse osseuse (Liang *et al.* 1992; Roholl *et al.* 1994; Quarto *et al.* 1995; Bergman *et al.* 1996; Kotev-Emeth *et al.* 2000; Chen 2004). Toutefois, l'importance du Mg^{2+} au niveau des fonctions des

cellules osseuses et du remodelage osseux est peu connue. Globalement, le Mg^{2+} est nécessaire à de multiples fonctions cellulaires, en tant que cofacteur d'enzymes intervenant entre autre dans les réactions métaboliques et dans la synthèse protéique (Romani et Scarpa 2000). Certaines études ont aussi indiqué qu'une réduction du Mg^{2+} extracellulaire entraîne une inhibition de la synthèse d'ADN favorisant de ce fait un arrêt de la croissance. À cet égard, il a été suggéré que certains canaux de la famille «melastatin related transient receptor potential» (TRPM) seraient responsables de l'homéostasie du Mg^{2+} cellulaire. Entre autre, le canal TRPM7 est reconnu pour être perméable au calcium (Ca^{2+}) et au Mg^{2+} et son activité serait modulée par le Mg^{2+} intracellulaire ce qui en ferait un joueur important dans la prolifération cellulaire.

Les traitements actuels de l'ostéoporose visent à freiner la perte de la masse osseuse et stimuler le remodelage osseux afin de réduire le risque de fracture. Ainsi le développement de meilleurs traitements de l'ostéoporose nécessite une meilleure compréhension des mécanismes fondamentaux de la régulation du processus de remodelage osseux afin de découvrir de nouvelles cibles thérapeutiques. Afin de mieux comprendre les mécanismes de régulation de l'homéostasie du Mg^{2+} dans la formation osseuse, mon projet de recherche vise à déterminer l'importance du Mg^{2+} et l'implication des canaux TRPM7 au niveau de la prolifération, de la migration et de la différenciation des ostéoblastes.

CHAPITRE I

1. ÉTAT DES CONNAISSANCES

1.1. Le tissu osseux

L'os est un tissu conjonctif spécialisé formant, avec le cartilage, le squelette. Il est constitué de cellules et d'une matrice extracellulaire qui a la particularité d'être minéralisée. Le tissu osseux est mis en place dès la période fœtale et reste sous le contrôle de facteurs hormonaux et locaux pendant toute la croissance. Ce tissu assure trois fonctions : une fonction mécanique (support et site de fixation des muscles pour la locomotion), une fonction protectrice (des organes vitaux) et une fonction métabolique par sa capacité à emmagasiner et à libérer un certain nombre d'éléments minéraux, principalement du calcium et du phosphate, afin d'assurer l'homéostasie minérale. Ainsi, sa fonction métabolique est associée au fait que le tissu osseux est en renouvellement constant, processus désigné remodelage.

1.2. Le remodelage osseux

Le remodelage se caractérise par deux activités opposées : la dégradation (résorption) d'os ancien par les ostéoclastes et la formation d'os nouveau par les ostéoblastes. Ce processus permet ainsi le maintien et le renouvellement permanent de la matrice osseuse. Ces deux activités qui définissent le remodelage osseux sont

étroitement couplées. En effet ce processus est complexe et est régulé de façon indépendante au niveau de chaque unité fonctionnelle de remodelage ou les ostéoclastes et les ostéoblastes sont étroitement associés (résorption puis formation). Il se caractérise par la succession de cinq phases (figure 1) (Aubin 2001). Une phase d'activation durant laquelle les précurseurs mononucléés des ostéoclastes prolifèrent et fusionnent au niveau d'une zone particulière de la surface osseuse et débutent la résorption. Cette phase est initiée par des facteurs systémiques et locaux agissant directement sur les ostéoclastes et leurs précurseurs. Cependant, la nature des facteurs et les mécanismes qui activent les préostéoclastes ne sont pas entièrement connus (voir section 1.3). Cette phase d'activation est suivie d'une phase de résorption, pendant laquelle les ostéoclastes résorbent la matrice minéralisée, creusant ainsi des lacunes de résorption. Cette dernière est suivie d'une phase d'inversion, pendant laquelle les précurseurs ostéoblastiques prolifèrent et se différencient en ostéoblastes matures. Le recrutement de ces cellules ostéogéniques serait dû à la libération de facteurs cellulaires et matriciels durant la phase de résorption. Finalement une phase de formation s'ensuit, pendant laquelle les ostéoblastes comblent les lacunes de résorption en apposant une nouvelle matrice collagénique (tissu ostéoïde). Certaines de ces cellules meurent par apoptose. D'autres ostéoblastes prennent un aspect aplati le long de la nouvelle matrice, et sont dites bordantes. Ces dernières sont très actives et assurent la minéralisation de la matrice osseuse. A la fin de leur activité de synthèse, les cellules bordantes deviennent inactives et la surface osseuse retourne alors à un état latent. Certains ostéoblastes seront englobés dans une logette appelée ostéoplaste dans la matrice osseuse et deviennent des ostéocytes et sont reliés entre eux et aux ostéoblastes par des jonctions cellulaires (Franz-Odentaal *et al* 2005). Ainsi dispersés dans la matrice osseuse, les ostéocytes détectent les microlésions et contrôlent le processus de remodelage osseux. Il est à noter que cette activité de formation dépend davantage du nombre d'ostéoblastes que de l'activité propre de chaque cellule (Aubin 2001; Aubin et Bonnelye 2000).

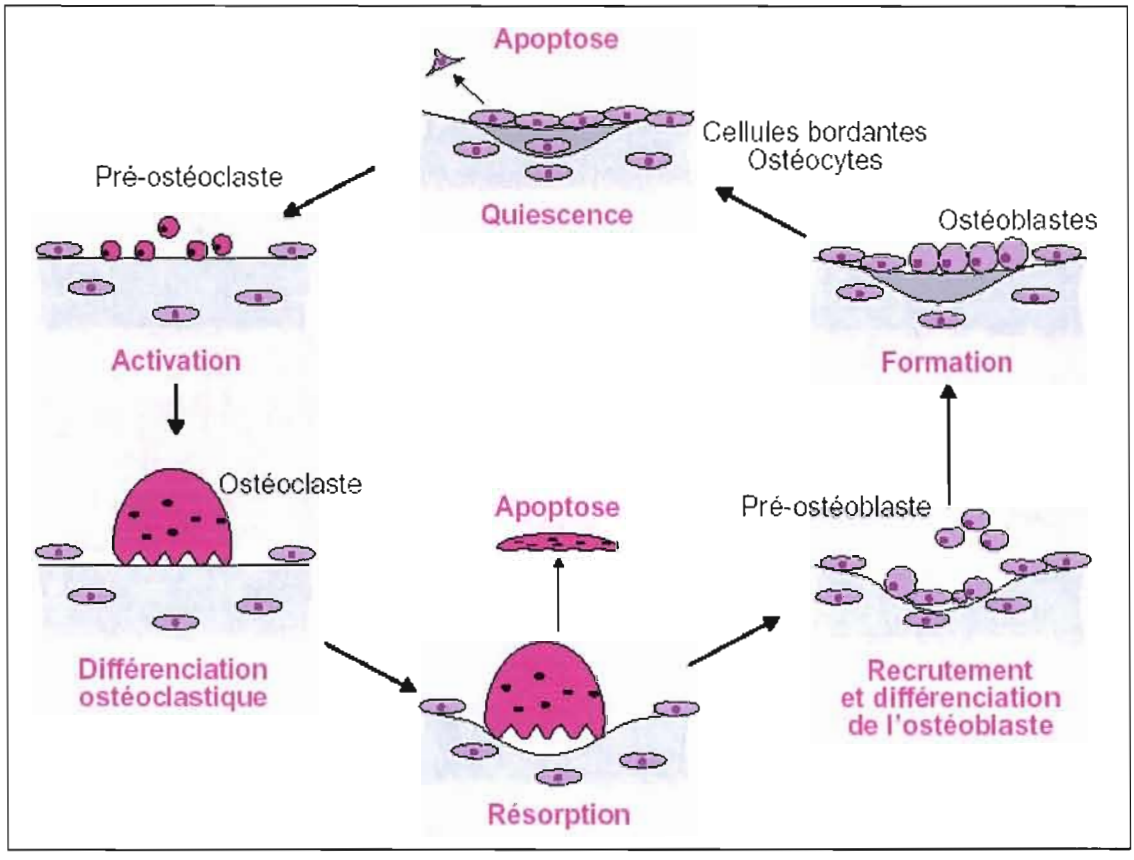


Figure 1. Le remodelage osseux.

Le cycle du remodelage débute par une phase d'activation, caractérisée par la différenciation des ostéoclastes, suivie d'une phase de résorption de la matrice par les ostéoclastes. À la suite de cette phase, les ostéoclastes se détachent et meurent par apoptose, puis les précurseurs des ostéoblastes se différencient en pré-ostéoblastes puis en ostéoblastes qui synthétisent une nouvelle matrice comblant la lacune de résorption. À la fin de cette phase de formation, les ostéoblastes deviennent des cellules bordantes, subissent un phénomène d'apoptose ou se laissent inclure dans la matrice en devenant des ostéocytes (Pierre Marie 2001).

1.3. Les cellules osseuses

Puisque le remodelage osseux dépend de l'action combinée des ostéoclastes et des ostéoblastes, l'intégration et la coordination des fonctions de ces deux types cellulaires sont nécessaires afin de maintenir une formation osseuse adéquate, pour procurer les fonctions mécaniques de l'os, la protection des organes et assurer l'homéostasie du calcium sérique de l'organisme.

1.3.1. Les ostéoclastes

Les ostéoclastes sont issus de la fusion de précurseurs hématopoïétiques de la lignée macrophage-monocyte, et ces derniers se différencient en ostéoclastes matures sous l'influence de facteurs de croissance et de cytokines (macrophage colony-stimulating factor (M-CSF), Receptor Activator of Nuclear faktor kappa B ligand (RANK-L)) et acquièrent la capacité unique de résorber la matrice calcifiée de l'os (Li *et al* 2002). Les ostéoclastes sont des cellules géantes (de 50 à 200 micromètres de diamètre), multinucléées (de 4 à 20 noyaux) et d'une durée de vie très courte (2 à 3 semaines). Elles sont polarisées, leurs noyaux sont apicaux et ont un appareil de Golgi très abondant et de nombreuses mitochondries et lysosomes. L'attachement de l'ostéoclaste à la matrice osseuse se fait par l'intermédiaire de podosomes et délimite un micro-compartiment étanche. Puis l'ostéoclaste induit via une pompe à protons une acidification du micro-compartiment pour la dissolution de la phase minérale de la matrice osseuse. De plus, l'ostéoclaste synthétise et sécrète plusieurs types d'enzymes impliqués dans la dégradation de la matrice osseuse. La dégradation entraîne la libération de facteurs de croissance inclus dans la matrice extracellulaire lors de la formation osseuse et notamment du «transforming growth factor ß» (TGF-ß), qui active le recrutement des préostéoblastes (Bonewald et Dallas 1994) et leur

accumulation à proximité de la zone de résorption. De plus, la libération dans le micro-compartiment du Ca^{2+} lors de la résorption pourrait aussi contribuer à l'inhibition de la différenciation et de l'activité ostéoclastique (Kanatani *et al* 1999).

1.3.2. Les ostéoblastes

Les ostéoblastes sont responsables de la production des constituants de la matrice osseuse et de sa minéralisation (Zhao *et al* 2000). La matrice osseuse est constituée en majorité de collagène de type I, en plus de protéoglycanes et de glycoprotéines (ostéopontine et ostéocalcine). Cette matrice non encore calcifiée porte le nom d'ostéoïde. Le collagène de type I est une protéine fibrillaire qui se compose de trois chaînes protéiques, se liant entre elles pour former une triple hélice. Après 10 jours, l'ostéoïde se minéralise, le Ca^{2+} et le phosphate formant ensemble les cristaux d'hydroxyapatite ($[Ca_{10}(PO_4)_6(OH)_2]$) qui se déposent entre les fibres de collagène, ce qui confère à l'os sa résistance à la rupture et à l'étirement. Les ostéoblastes ont pour origine les cellules souches mésenchymateuses présentes principalement dans le stroma médullaire, mais que l'on peut retrouver aussi au niveau du périoste (membrane qui couvre la surface externe du tissu osseux compact) et de l'endoste (couche de tissu qui tapisse la cavité interne (médullaire) des os longs et qui renferme la moelle) (Figure 2). Ces cellules ostéogéniques du stroma médullaire proviennent de la prolifération clonale de cellules souches pluripotentes pouvant donner naissance à des clones de cellules adipeuses mésenchymateuses ou chondroblastiques après induction par des facteurs hormonaux et locaux. Ceci suggère l'existence d'un précurseur commun aux chondroblastes, ostéoblastes, adipocytes, myoblastes et fibroblastes (Zhao *et al* 2000 ; Marie 1999). La différenciation des précurseurs cellulaires vers la voie ostéoblastique se caractérise premièrement par une phase de prolifération cellulaire associée à l'expression de

gènes précoces (oncogènes *fos* et *myc*, histone H4), et puis d'une phase de maturation cellulaire caractérisée par l'induction de gènes associés à la production de la matrice extracellulaire (collagène de type 1, fibronectine, ostéopontine), puis à sa minéralisation (sialoprotéine osseuse, ostéocalcine) (Franz-Odendaaal *et al* 2005). Les ostéoblastes différenciés sont des cellules cuboïdales alignées le long de la matrice osseuse. Leur cytoplasme, basophile, est riche en réticulum endoplasmique granulaire, en mitochondries et présente un appareil de Golgi très développé, démontrant une activité de synthèse très importante.

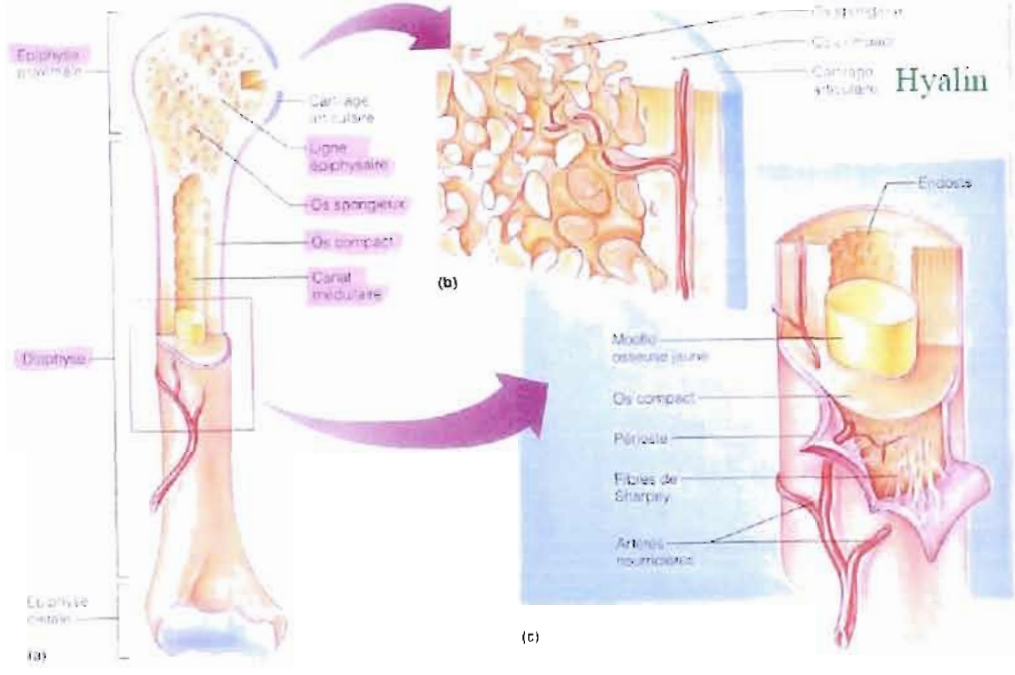


Figure 2. Organisation du tissu osseux. a) Structure générale d'un os long. b) Grossissement de l'épiphyse et structure trabéculaire de l'os. c) Grossissement de la diaphyse et organisation du cortex osseux. Tiré de Biologie humaine : anatomie et physiologie par Elaine N.Marieb, René Lachaine. Sixième édition page 114.

1.3.2.1. Marqueurs phénotypiques des ostéoblastes

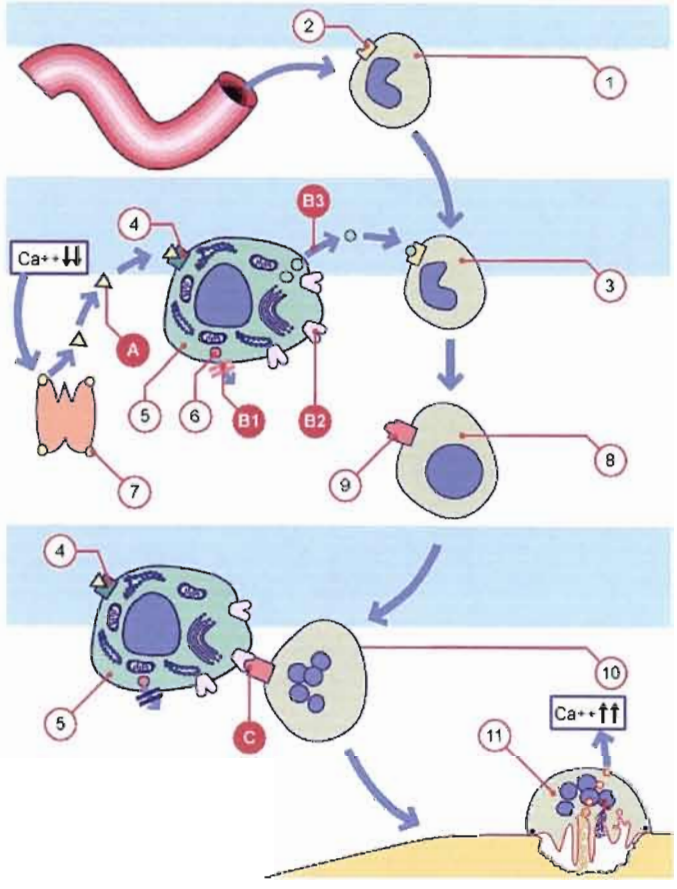
Plusieurs marqueurs caractéristiques de la différenciation ostéoblastique ont été identifiés. L'apparition d'une activité phosphatase alcaline est un marqueur qui apparaît dès le stade de précurseur ostéoblastique. Son activité permet l'hydrolyse des pyrophosphates inorganiques, qui sont des inhibiteurs de la calcification. Les ostéoblastes secrètent le collagène de type I et l'ostéopontine. L'ostéopontine est une phosphoglycoprotéine qui comporte une séquence RGD (arginine-glutamine-aspartate) et qui intervient dans la phase d'ancrage des ostéoclastes à la matrice osseuse minéralisée (Simonet *et al* 1997). Son degré de phosphorylation pourrait aussi moduler la mobilité des ostéoblastes à la surface de la matrice. De plus lorsqu'elle est phosphorylée, elle inhibe la formation des cristaux d'hydroxyapatite et pourrait donc réguler le processus de minéralisation de la matrice osseuse.
La sialoprotéine osseuse et l'ostéocalcine sont des marqueurs plus tardifs de la différenciation ostéoblastique et ne sont exprimés que par les ostéoblastes différenciés. L'ostéocalcine est dépendante de la vitamine K et cette protéine régule le dépôt calcique en liant le calcium (Hauschka *et al* 1978). En effet, cette protéine contient 3 résidus d'acides aminés (17, 21 et 24) dits dépendant de la vitamine K que l'on nomme acide gamma carboxyglutamique (Gla). Des études *in vitro* ont suggérés que l'ostéocalcine avait le rôle de limiter le processus de minéralisation et des études *in vivo* effectuées chez des souris dépourvues d'ostéocalcine ont montré une augmentation de la masse osseuse (Zhou *et al* 1994; Ducy *et al* 1996).

La sialoprotéine osseuse est une phosphoglycoprotéine également synthétisée par les ostéoclastes. Elle a les mêmes effets que l'ostéopontine sur les ostéoclastes, mais par contre favorise, *in vitro*, la formation et la nucléation des cristaux d'hydroxyapatite. L'ostéocalcine est spécifique du tissu osseux et y existe en quantité abondante, représentant 15 à 25 % des protéines non collagéniques de l'os. Elle

semble avoir un rôle chimiotactique pour les ostéoclastes et favoriser l'adhésion et l'étalement de ces cellules (Gehron 1989).

1.3.2.2. Régulation de la résorption osseuse par les ostéoblastes

En plus de leur implication dans la formation du tissu osseux, les ostéoblastes régulent l'activité des ostéoclastes (figure 3). Les précurseurs de la famille des ostéoclastes-macrophages [1, fig. 3] quittent les vaisseaux sanguins et arrivent à proximité d'un ostéoblaste. Le «macrophage colony stimulating factor» (M-CSF) [B3, fig. 3] sécrété par les ostéoblastes [5, fig. 3] se lie à son récepteur [2, fig. 3] à la surface du précurseur d'ostéoclaste [3, fig. 3], ce qui cause sa transformation en précurseur d'ostéoclaste immature [8, fig. 3] pourvu maintenant à sa surface d'un «Receptor activator of nuclear factor kappa B» (RANK) [9, fig. 3]. Par la suite, le précurseur d'ostéoclaste immature se lie par son récepteur RANK au «Receptor Activator of Nuclear factor B ligand» (RANK-L) présent à la surface des ostéoblastes [B2, fig. 3]. L'ostéoclaste immature [10, fig. 3] est ainsi transformé en ostéoclaste mature [11, fig. 3]. La maturation des ostéoclastes est terminée lorsque la zone d'ancrage et la bordure plissée sont totalement formées.



1. Précurseur de la famille des ostéoclastes-macrophages
2. Ligand M-CSF (macrophage-colony stimulating factor)
3. Macrophage
4. Récepteur de parathormone
5. Ostéoblaste
6. Ostéoprotégérine (glycoprotéine)
7. Les 4 glandes parathyroïdes autour de la thyroïde (dessiné à une plus petite échelle)
8. Précurseur d'ostéoclaste
9. Récepteur RANK
10. Ostéoclaste immature
11. Ostéoclaste activé

Figure 3. Régulation de la différenciation des ostéoclastes par les ostéoblastes.

Afin d'assurer une régulation de la différenciation des ostéoclastes, les ostéoblastes sécrètent une glycoprotéine désignée ostéoprotégérine (OPG) [6] pouvant lier le RANKL à la surface des ostéoblastes et prévenir ainsi sa liaison au RANK à la surface des ostéoclastes immatures. Ainsi, le rapport d'expression de l'OPG et du RANKL déterminera le niveau de différenciation des ostéoclastes (Simonet *et al* 1997; Gehron *et al* 1993).

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1.4. Contrôle du remodelage osseux

L'os est un très important réservoir de Ca^{2+} pour tous les processus biologiques dépendants du Ca de l'organisme. La concentration de Ca^{2+} totale dans le liquide extracellulaire doit être maintenue constante (environ 2.5 mmol/l). Lorsque la concentration en Ca^{2+} du milieu extracellulaire chute, il y a activation des ostéoclastes, ce qui favorise la dégradation du tissu osseux et ainsi la mobilisation de Ca^{2+} vers la circulation (figure 3). L'hormone principalement responsable de ce mécanisme est la parathormone (PTH) [A, fig. 3] sécrétée par les glandes parathyroïdes [7, fig. 3]. Sa sécrétion est directement régulée par la concentration de Ca^{2+}circulant. Quand la concentration extracellulaire de Ca chute, la parathyroïde est alors stimulée et sécrète la PTH. Cette hormone se lie ensuite à son récepteur à la surface des ostéoblastes [4, fig. 3] ce qui entraîne une augmentation de la sécrétion de M-CSF, une inhibition de la synthèse de l'OPG et une augmentation de l'expression de RANK-L par les ostéoblastes. Ainsi, la PTH favorise la différenciation des ostéoclastes et la résorption osseuse. D'un autre côté lorsque la concentration du Ca^{2+} en milieu extracellulaire augmente, il y a stimulation de la sécrétion de l'hormone calcitonine. Cette hormone se lie ensuite à son récepteur à la surface des ostéoclastes ce qui entraine une diminuation de la bordure en brosse et une inhibition de la dégradation de l'os (Simonet *et al* 1997; Cohen-SolalM et deVernejoul 2003). Ainsi, l'hormone PTH favorise l'ostéolyse ce qui permet la libération de Ca^{2+} dans le sang tandis que la calcitonine inhibe les ostéoclastes, ce qui diminue la résorption osseuse et donc augmente le stockage du Ca^{2+} dans l'os.

1.5. Contrôle de l'ostéogenèse

L'activité de la formation osseuse dépend principalement du nombre et de l'activité de cellules ostéoblastiques (Rubin et Koide 1976). Un grand nombre

d'hormones et de facteurs de croissance agissent au niveau du recrutement et de la prolifération des cellules ostéoblastiques et jouent donc un rôle essentiel dans le contrôle de la formation osseuse. Les hormones peuvent avoir soit une action directe via des récepteurs spécifiques, soit une action indirecte en augmentant la synthèse de facteurs locaux par les ostéoblastes. Ainsi la 1,25-dihydroxyvitamine D_3 ($1,25(OH)_2D_3$), mais également les œstrogènes et l'hormone de croissance régulent la formation osseuse (Strewler 2001). L'ostéogenèse est aussi régulée par de nombreux facteurs de croissance produits par les cellules médullaires ou les ostéoblastes eux-mêmes. Certains de ces facteurs de croissance sont systémiques, d'autres sont locaux et peuvent de plus être incorporés dans la matrice osseuse (Christakos 1996). Parmi ces facteurs de croissance, on peut citer les "Fibroblast Growth Factor " (FGF), les "Transforming Growth Factor ß" (TGF ß) et "l'Insulin-Like Growth Factor" (IGF). Il est intéressant de noter que bon nombre de ces facteurs induisent une réponse calcique. La signalisation calcique est devenue un champ d'étude qui établit le Ca^{2+} intracellulaire comme étant un second messager clé dans le contrôle de plusieurs fonctions cellulaires.

1.6. Le calcium et les fonctions cellulaires

L'ion calcium est considéré comme un second messager contrôlant un large spectre de processus biologiques tels la contraction musculaire, la sécrétion, le métabolisme, l'excitabilité neuronale et musculaire, la différenciation, la prolifération ainsi que la mort cellulaire (apoptose) (Bootman *et al* 2001), si bien que la régulation de la concentration intracellulaire de ce cation est d'une importance primordiale. La concentration du Ca^{2+} libre cytosolique est maintenue entre 50 et 100nM ce qui est 20 000 fois inférieur à la concentration de Ca^{2+} extracellulaire. Pourtant la concentration en Ca^{2+} à l'intérieur du réticulum endoplasmique est très élevée, de

l'ordre de 100 µM. La signalisation cellulaire par le Ca^{2+} est toujours liée à une augmentation de sa concentration cytosolique. Cette concentration élevée est nécessaire à l'activation des protéines liant le Ca^{2+}, tel que la calmoduline et la troponin C. De plus le signal calcique est caractérisé par son origine, son amplitude, sa durée (de quelques microsecondes à plusieurs heures) et aussi par sa fréquence. Plusieurs facteurs modifient la concentration cytosolique en Ca^{2+}. Ce dernier est mobilisé du milieu extracellulaire (processus d'influx) par l'activation de canaux ioniques à la membrane plasmique (Wes *et al* 1995) ou par une libération à partir de réserves internes, principalement de réticulum endoplasmique (Putney 1986).

La cellule maintient un faible niveau de Ca^{2+} intracellulaire (effet tampon) grâce à l'existence de protéines capables de fixer le Ca^{2+} ``calcium-binding proteins``. (Clapham 2007). Cependant, lors d'une stimulation, la cellule déclenche une cascade de signalisation, entraînant en premier lieu la libération du Ca^{2+} emmagasiné dans les réserves intracellulaires tel le réticulum endoplasmique et en deuxième lieu une activation des canaux membranaires (canaux ioniques) ce qui favorise un influx calcique et augmentera la concentration de Ca^{2+} dans le cytosol. Par la suite, le Ca^{2+} se lie à des protéines liantes spécifiques pour les activer et celles-ci à leur tour activent un certain nombre de protéine kinases. Les mécanismes favorisant l'augmentation du Ca intracellulaire sont contrebalancés par l'activité des pompes : la ``Plasma Membrane $Ca^{2+}$ ATPase`` (PMCA) qui fait sortir le Ca^{2+} de la cellule et la pompe ``Sarco(Endo) plasmic Reticulum $Ca^{2+}$ ATPase`` (SERCA) qui fait entrer le Ca^{2+} dans les organelles, ce qui diminuent la concentration de Ca^{2+} dans le cytosol et permettent de regarnir les réserves intracellulaires (Clapham 2007).

Les hormones et les facteurs de croissances vont se lier à leurs récepteurs spécifiques à la surface des cellules et déclencher une série d'événements intracellulaires, dont certains d'entre eux vont mener à l'élévation de la concentration intracellulaire de Ca^{2+}. Si l'hormone se lie à un récepteur de la famille des récepteurs

couplés aux protéines G, cette dernière va activer une protéine G hétérotrimérique de la famille Gq/11 (Hubbard et Hepler 2006). La sous-unité alpha de la protéine Gq/11, de même que son complexe beta/gamma, peuvent activer la phospholipase C beta. Si l'hormone se lie à un récepteur de la famille des récepteurs tyrosine kinase, cette dernière va directement activer une phospholipase C gamma.

Les phospholipases C beta et gamma vont hydrolyser un lipide de la membrane cytoplasmique, le phosphatidylinositol 4,5-bisphosphate, pour produire l'inositol 1,4,5-trisphosphate (IP_3) et le diacylglycérol. L'IP_3 va alors diffuser dans le cytosol pour se lier et activer son récepteur-canal situé au niveau du réticulum endoplasmique et initier la première phase de la signalisation calcique (Malathi *et al* 2003). Comme la concentration du Ca^{2+} dans le réticulum endoplasmique est au moins 10 000 fois supérieure à la concentration du Ca dans le cytosol, l'ouverture du canal va permettre au Ca^{2+} de passer du réticulum endoplasmique vers le cytosol. Cependant, le contenu en Ca^{2+} dans le réticulum endoplasmique est très limité et, dû à l'activité des pompes calciques et échangeurs ioniques situés à la membrane cytoplasmique, la concentration de Ca^{2+} intracellulaire revient au niveau basal dans quelques minutes.

Ainsi, pour maintenir une fonction cellulaire à plus long terme, il se produit une entrée de Ca^{2+} de l'extérieur de la cellule vers le cytosol. Cette entrée de Ca^{2+} constitue la seconde phase de la signalisation calcique et est due à l'activation des canaux TRPC ``transient receptor potential canonical`` et Orai1. La signalisation calcique est terminée une fois que l'hormone se dissocie de son récepteur, cessant la production d'IP_3, qui est rapidement dégradé, suivi par la fermeture du récepteur-canal de l'IP_3. L'entrée de Ca^{2+} cesse une fois que le réticulum endoplasmique retrouve sa concentration de Ca^{2+} initiale (Abdel-Latif 1986).

L'augmentation du Ca^{2+} dans le cytosol est capable de provoquer l'expression de certains gènes codant pour des facteurs de transcription. Entre autres, une augmentation de la concentration intracellulaire de Ca^{2+} entraîne l'activation de la

cascade de signalisation des «mitogen-activated protein kinases» (MAPK) (Lipskaia et Lompre 2004; Malathi *et al* 2003) ainsi que la possibilité d'une signalisation croisée impliquant la voie phospholipase C (figure 4).

Le Ca^{2+} en tant que second messager intracellulaire joue un rôle primordial dans l'induction du processus de mort cellulaire. L'apoptose ou mort cellulaire programmée est un des deux mécanismes, avec la nécrose, menant à la mort cellulaire. Une accumulation incontrôlée du Ca^{2+} cytosolique est toxique pour la cellule, le Ca^{2+} pouvant favoriser la précipitation du phosphate qui est important à plusieurs niveaux pour la cellule, et entraîne une induction des facteurs de transcription directement impliqués dans le processus de la mort cellulaire programmée par apoptose. Entre autres, une augmentation locale de Ca^{2+} peut entraîner une augmentation de la concentration de Ca^{2+} mitochondriale, induire la libération de cytochrome c, et activer la voie apoptotique (Jayaraman et Marks 1997).

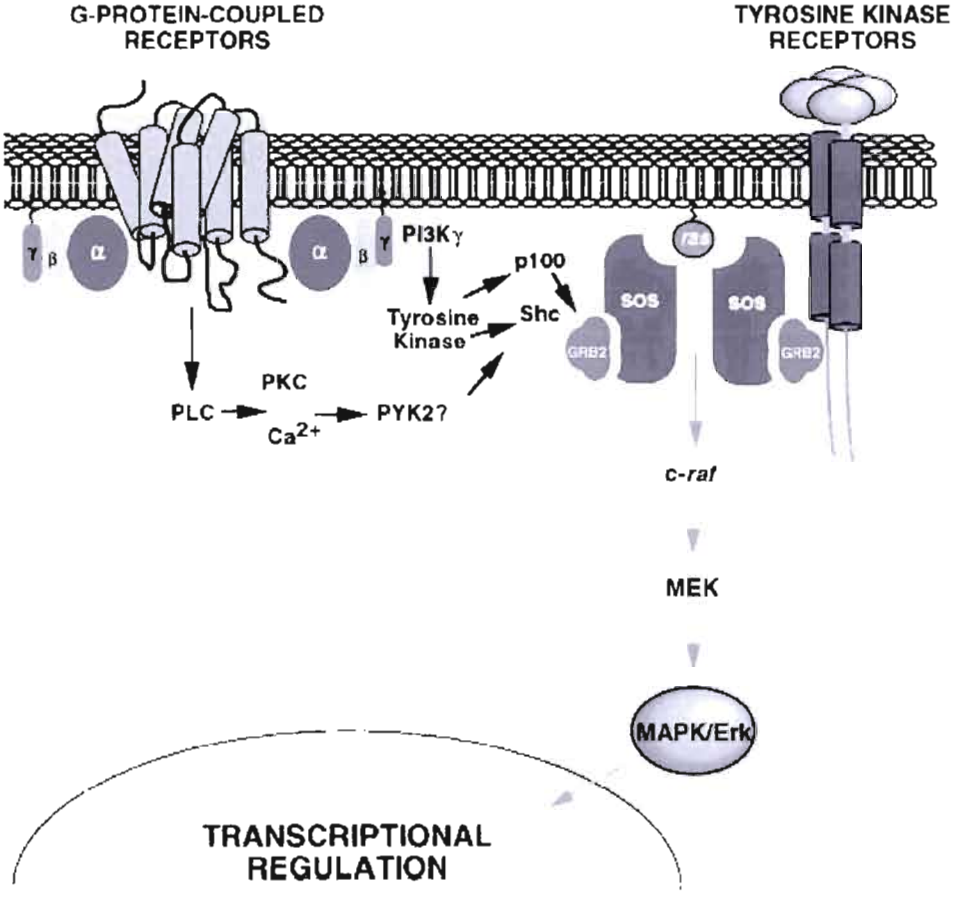

Figure 4 : Cascades de signalisation initiées par des récepteurs de la membrane plasmique chez les mammifères. L'activation de récepteurs couplés à des protéines G ou des récepteurs tyrosine kinase entraîne l'activation de cascades de phosphorylation, notamment la voie des MAPK, soit de façon directe ou par réaction croisée impliquant la phospholipase C (PLC). Tiré de Lopez-Ilasaca M 1998.

1.7. Les canaux calciques

Le Ca^{2+} entre dans la cellule par plusieurs types des canaux calciques. Ces derniers forment des pores permettant le passage rapide et sélectif d'ions Ca^{2+} au travers de la membrane plasmique (Nargeot et Charnet1994). Le flux d'ions au travers de ces canaux, qui est fonction du gradient électrochimique, créé un courant calcique. Les canaux calciques activés par la dépolarisation membranaire, tout comme les canaux sodiques et potassiques forment une superfamille de protéines. On distingue souvent ceux qui sont voltage-dépendants (l'ouverture du canal se produit lorsque le potentiel de membrane atteint une certaine valeur) de type T (transitoires) ou de type L, P/Q, N, et R (longue durée) et ceux qui sont ligand-dépendants (l'ouverture du canal est déclenchée par une molécule) tels les canaux TRP ``transient receptor potential``.

1.7.1. Les canaux sensibles au voltage

La famille des canaux calciques voltage-dépendants (CCDV) comporte deux classes : (1) les canaux à « bas seuil d'activation » (BSA) activés par de faibles dépolarisations membranaires regroupent exclusivement les canaux de type T et (2) les canaux à « haut seuil d'activation » (HSA) activés pour de plus fortes dépolarisations membranaires regroupent les canaux de type L, P/Q, N, et R (Catterall 2000). Ce sont des canaux formés de cinq sous unités : α_1, α_2, β, δ et γ; la sous unité α_1 formant le pore. Cette dernière est composée de quatre domaines (I à IV) chacun d'eux étant formé de six hélices transmembranaires et d'une boucle associée à la membrane entre les segments S5 et S6 (Catterall 2000). Or, la conformation du

canal amène la formation du pore par l'association des boucles des domaines I à IV de la sous unité α_1 (Fig.5). Les canaux voltage-dépendants ont une importance marquée dans les cellules excitables, par contre leur rôle dans les cellules non excitables telles que les ostéoblastes est encore obscur. (Tableau 1)

	Sous-unité α_1	Courant	Pharmacologie	Localisation
Haut seuil d'activation (HSA)	α_{1S} ($Ca_v1.1$)			Muscle squelettique
	α_{1C} ($Ca_v1.2$)	Type L	Dihydropyridines (par exemple, nifédipine) Phénylalkylamines Benzothialzépines	Neurones, cœur, muscles lisses, cellules endocrines
	α_{1D} ($Ca_v1.3$)			Neurones, cœur, cellules endocrines
	α_{1F} ($Ca_v1.4$)			Rétine
	α_{1A} ($Ca_v2.1$)	Type P/Q	ω-Agatoxine IVA ω-Agatoxine IIIA ω-Conotoxine MVIIC	Neurones, cellules pancréatiques β
	α_{1B} ($Ca_v2.2$)	Type N	ω-Conotoxine GVIA ω-Conotoxine MVIIA ω-Agatoxine IIIA	Neurones
	α_{1E} ($Ca_v2.3$)	Type R	Nickel, Cadmium	Neurones, cellules endocrines
Bas seuil d'activation (BSA)	α_{1G} ($Ca_v3.1$)	Type T	Benzimidazoles (par exemple, mibéfradil) Amiloride Éthosuximide Kurtoxine Nickel	Neurones, cœur, muscles lisses, cellules endocrines, spermatozoïdes
	α_{1H} ($Ca_v3.2$)			Neurones, cœur, foie, muscles lisses, cellules endocrines
	α_{1I} ($Ca_v3.3$)			Neurones, spermatozoïdes

Tableau I. *Classification moléculaire, pharmacologique et localisation tissulaire des canaux calciques dépendants du voltage.*

Tableau 1 : classification moléculaire des canaux calciques dépendants du voltage. (Norbert Weiss et Michel De Waard 2006).

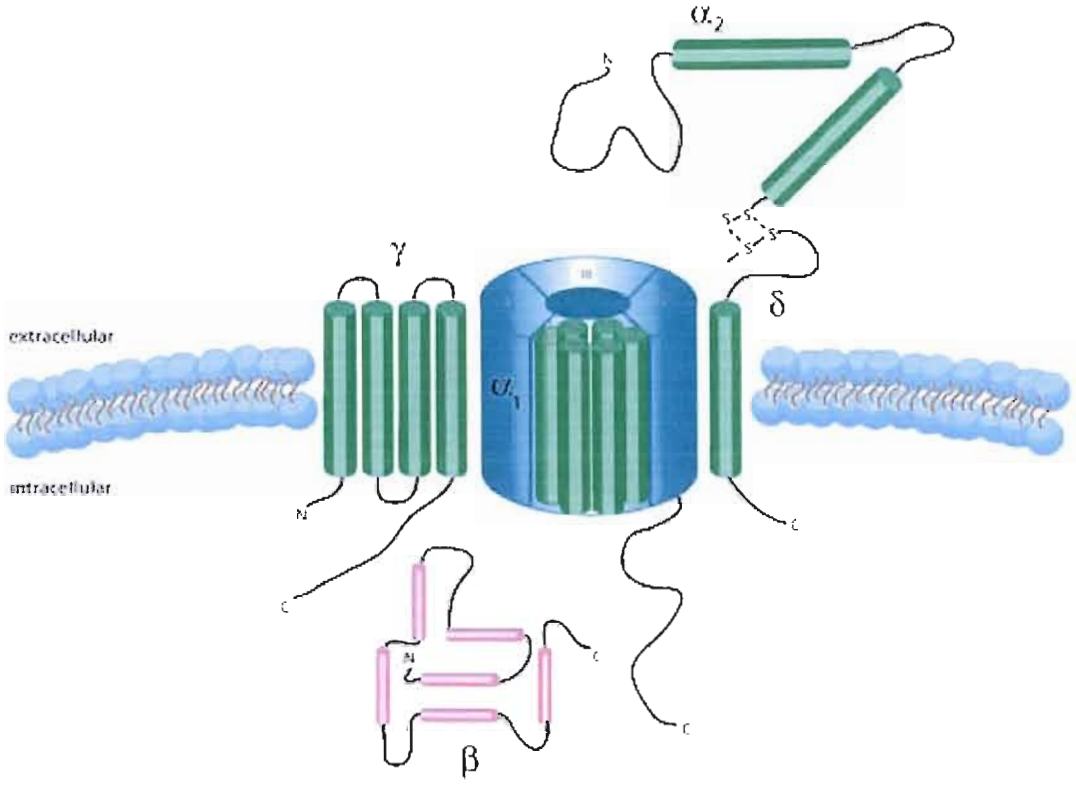

Figure 5. Organisation membranaire des canaux calciques dépendants du voltage. (Tiree de http://www.sigmaaldrich.com/life-science/cell-biology/learning-center/pathway-slides-and/voltage-gated-calcium-channel.html)

Les CCDV (HSA et BSA) se composent d'une sous-unité principale α1 (en bleu) formant le pore ionique. Elle se compose de quatre domaines (domaines I à IV) constitués chacun par six segments transmembranaires (S1 à S6). Ces quatre domaines sont reliés entre eux par des boucles cytoplasmiques reliant les domaines I à II (boucle I-II), II à III (boucle II-III) et III à IV (boucle III-IV). Les segments S4, riches en résidus basiques arginine et lysine, constituent le senseur de voltage. Les

boucles extracellulaires et transmembranaires reliant les segments S5 et S6 (boucles P, en rouge) forment le pore ionique. (Yamakage et Namiki 2002).

1.7.2. Les canaux TRP («transient receptor potential»)

Les canaux TRP ont été identifiés et caractérisés chez la drosophile. La rétine de Drosophile répond normalement par une dépolarisation suite à une stimulation lumineuse. Lors des études d'un mutant, cette réponse était transitoire et se caractérisait par une cécité. Le mutant a donc été appelé TRP pour «transient receptor potential» (Pedersen *et al* 2005). Les canaux TRP chez la drosophile sont des canaux perméables au Ca^{2+} et sensibles à la lumière, et leur activation est intimement liée à l'activation de la PLC et à la libération de Ca^{2+} du réticulum endoplasmique. Les canaux TRP sont devenus de ce fait les prototypes parfaits des canaux activés par la déplétion des réserves de Ca^{2+} intracellulaire (Pedersen *et al* 2005). Par la suite de nombreux canaux apparentés ont été identifiés. Récemment, les canaux TRP ont été regroupés en 7 familles chez la drosophile : TRPC1-7 Canonique, TRPV1-6 Vanilloïde, TRPM1-8 Mélastatine, TRPP1-3 Polycystine, TRPML Mucolipine, TRPA Ankyrine et les TRPN (nompC) (Vassort et Alvarez 2009). Chez les mammifères, les TRPN ne sont pas représentés.

La structure des canaux de la superfamille des TRP est constituée de six segments transmembranaires (S1 à S6) et d'un pore entre les cinquième et sixième segments transmembranaires, structure semblable à celle des canaux potassiques /(Figure 6). Le canal TRP est formé par l'association de quatre sous unités identiques (canal homotétramérique) ou différentes (canal hétérotétramérique). Les deux

familles TRP-C et TRP-V présentent dans la partie N-terminale entre deux et quatre motifs ankyrines. Plusieurs fonctions ont été attribuées à ces motifs ankyrines notamment dans l'interaction protéine-protéine, suggérant une interaction avec des éléments de certaines cascades de signalisation et aussi avec la calmoduline.

Jusqu'à maintenant, l'expression des TRP a été démontrée dans plusieurs organes tels le cerveau, le cœur, les poumons, les reins, le foie, la rate, les intestins, les testicules, les ovaires, l'utérus et le placenta, ainsi que dans plusieurs types cellulaires (Pederson *et al* 2005). Leur rôle est essentiel pour que l'organisme intègre les informations issues du monde extérieur et au niveau cellulaire pour détecter les caractéristiques de l'environnement (Jordt et Ehrlich 2007). Cependant, l'expression des canaux TRP ainsi que leurs fonctions au niveau des cellules osseuses et du remodelage osseux n'est pas connue.

Ainsi la compréhension des fonctions des canaux TRP sur l'activité des cellules ostéoblastiques est inportante afin de mieux comprendre l'effet des canaux TRP sur le métabolisme osseux.

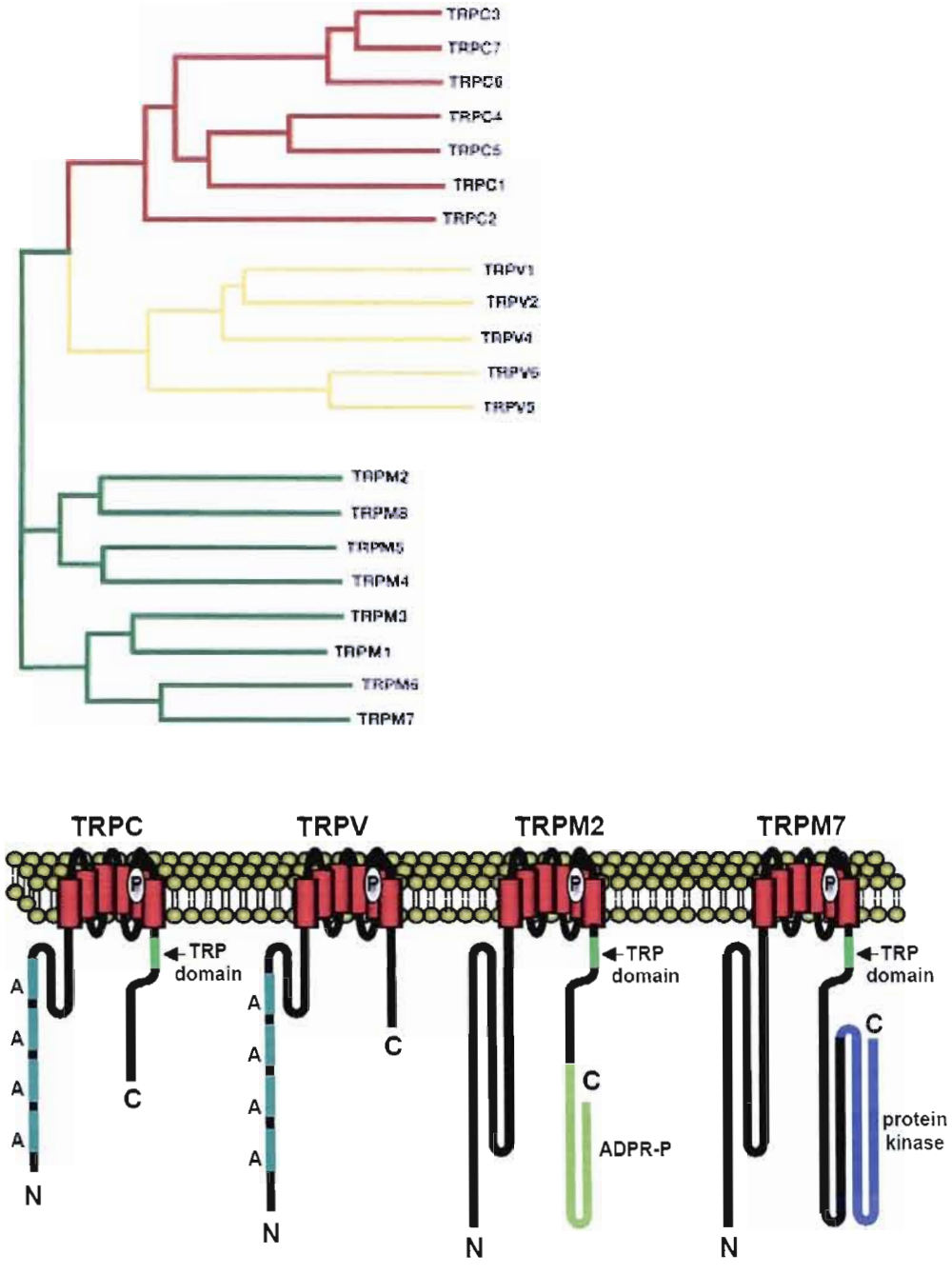

Figure 6. Diversité moléculaire des canaux TRP (Clapham *et al* 2001).

1.7.2.1 Les canaux TRPC canoniques

Plusieurs études rapportent que les canaux TRPC sont activés en réponse à une stimulation des récepteurs couplés à la phospholipase C (PLC), et sont notamment modulés par la calmoduline et les protéines fixant le Ca (Kiselyov *et al* 2005). Les membres de cette famille sont classés en 4 groupes selon leur homologie de séquence : TRPC1, TRPC2, TRPC3/6/7 et TRPC4/5.

1.7.2.1.1. Activation des canaux TRP-C

Ces canaux sont tous activés en réponse à une stimulation des récepteurs activant diverses isoformes de la phospholipase C, PLC, et sont notablement modulés par la calmoduline et diverses autres protéines fixant le Ca^{2+} (Zhu 2005). Typiquement les canaux TRPC3/6/7 sont activés plus spécifiquement par le diacylglycérol, DAG, tandis que le mécanisme d'activation des TRPC1/4/5 par la PLC reste controversé.

Le canal TRPC1 fut le premier TRP à être cloné chez les mammifères et a été identifié comme un canal sensible à l'étirement chez les vertébrés. TRPC1 pourrait être associé à Orai1 et former un complexe TRPC1-Orai1-STIM1 *(stromal interacting molecule)* contribuant à la fonction SOCC (Ambudkar *et al* 2007). Large (2002) a rapporté que les canaux TRPC6 et TRPC3 étaient des éléments essentiels de l'activation des canaux cationiques non sélectifs activés par la stimulation α_1-adrénergique dans le muscle lisse de la veine porte du lapin et dans l'artère de l'oreille respectivement. Les canaux TRPC1 et TRPC6 seront activés par

l'angiotensine par des voies spécifiques de transduction du signal, respectivement dépendantes ou non de la proteine kinase C (PKC) (Large *et al* 2009). Pour les canaux TRPC3, l'insuline induit sa translocation à la membrane plasmique dans les cardiomyocytes ventriculaires de souris tandis que cet effet apparaît nettement réduit dans un modèle de souris diabétique, *ob/ob* (Fauconnier *et al* 2007).

1.7.2.2. Les canaux TRPV "Vanilloïde"

La famille des canaux TRPV comprend six membres formant 4 groupes : TRPV1/TRPV2, TRPV3, TRPV4, TRPV5/TRPV6. Ces canaux forment des complexes tétramériques et présentent dans la partie N-terminale de trois à cinq motifs ankyrines.

Les canaux TRPV1-4 sont tous activés par la chaleur (Sharif Naeini *et al* 2006) et jouent aussi un rôle important comme éléments sensibles à l'étirement (variation du volume cellulaire) dans tout le règne animal (O'Neil et Heller 2005). Les canaux TRPV1-4 présentent un rapport de perméabilité pour le Ca^{2+} et le Na^{+} (P_{Ca}/P_{Na}) de 1 à 10 tandis que les canaux TRPV5/TRPV6 sont séléctif aux ions Ca^{2+} et 100 fois plus perméables au Ca^{2+}. Le canal TRPV1 est activé par la capsaïcine, anandamide, divers eicosanoïdes, leucotriènes B4, N-arachidonoyl dopamine, adénosine et 2-amino ethoxyphénylborate (2-APB), le pH acide et une température modérée (> 43°C). Dans le muscle lisse vasculaire, le canal TRPV2 est un élément sensible à l'hypotonicité et

à l'étirement (Muraki *et al* 2003). Le camphre et 2-APB activent le canal TRPV3. Le canal TRPV4 est activé par l'α-phorbol, le 4α-phorbol, et le 12,13-didécanoate. Le canal TRPV4 est exprimé dans la plupart des tissus et de façon très abondante dans le rein. Ce canal est activé par l'hypotonicité *via* une phosphorylation sur le résidu tyrosine 253 par une tyrosine kinase (Xu *et al* 2003). Le canal TRPV5 est essentiel à la réabsorption du Ca dans le rein, et le canal TRPV6 assure la réabsorption du Ca^{2+} dans l'intestin. Les canaux TRPV5 et TRPV6 sont régulés par une inactivation dépendante du Ca^{2+} (demi-inactivation ≈ 100 nM). Ils sont aussi bloqués par le Mg^{2+}, propriété dépendante du résidu aspartate dans le filtre de sélectivité du pore du canal.

1.7.2.3. Les canaux TRPM ''Mélastatine''

Les TRP-M sont des protéines beaucoup plus grosses que les autres TRP (plus de 1000 et jusqu'à 2000 acides aminés contre 400 à 700 pour les autres familles). La famille des canaux TRPM comprend 8 membres, divisés en 4 sous-groupes selon les critères d'homologie de séquence: TRPM1/TRPM3, TRPM2/TRPM8, TRPM4/TRPM5, TRPM6/TRPM7 (Pedersen *et al* 2005).

Ces canaux ont été nommés après la découverte de la protéine TRPM1 dans les cellules de mélanomes. Une corrélation inverse existe entre son expression et la progression du mélanome. Tous les autres membres de cette famille TRPM sont aussi liés à des tumeurs humaines (Kraft et Harteneck 2005). Les membres de la famille

des canaux TRPM sont impliqués dans la tumorigenèse, la prolifération et la différenciation cellulaires.

Les canaux TRPM2, TRPM6 et TRPM7 forment des *''chanzymes''* (des canaux ioniques montrant une activité enzymatique). En effet, ils possèdent dans leurs extrémités carboxy-terminales une région à activité enzymatique qui intervient par un mécanisme d'autophosphorylation dans l'activation du canal (Scharenberg 2005). Le canal TRPM2 montre une activité ADP-ribose hydrolase associée au motif NUDT9 (*human nucleoside diphosphate linked moiety X-type motif 9*) de la zone carboxy-terminale. Le stress oxydatif active le canal TRPM2 en augmentant le largage d'ADP-ribose de la mitochondrie. La délétion du domaine NUDT9 supprime l'activation par le peroxyde d'hydrogène. De ce fait le canal TRPM2 serait un détecteur du statut redox cellulaire. Le canal TRPM3 est perméable au Ca^{2+} et sera activé par les variations d'osmolarité extracellulaire et par la déplétion des réservoirs calciques.

Les canaux TRPM4 et TRPM5 sont imperméables au Ca. Pourtant les canaux TRPM6, TRPM7, ainsi que des variants de TRPM3 ont une très forte perméabilité pour le Ca^{2+} et le Mg^{2+}. Les canaux TRPM4/TRPM5 sont perméables aux ions monovalents et sont activés par le Ca. Le canal TRPM4 est impliqué dans le contrôle de l'activité rythmique cardiaque et ses perturbations (Demion *et al* 2007). La dégradation du phosphatidylinositol 4,5-bisphosphate (PIP_2) lors de l'activation des PLC par certains médiateurs (α_1-adrénergique, muscarinique, angiotensine, etc) inhibe l'activité de TRPM4 (Nilius *et al* 2006). Le canal TRPM5 est essentiel pour la transduction des goûts sucré et amer. Le canal TRPM8 est activé par le froid et les

agents pharmacologiques donnant cette sensation comme le menthol. Les études sur des souris transgéniques déficientes en TRPM8 montrent que ce canal est le principal détecteur du froid (Bautista *et al* 2007).

Les canaux TRPM6 et TRPM7 assurent l'homéostasie du Mg^{2+}. Ils présentent une forte perméabilité pour cet ion tout en étant régulés par sa concentration intracellulaire. En relation avec le sujet de la présente thèse, il est intéressant de noter que des études épidémiologiques ont établi un lien entre l'apport en Mg^{2+} et la densité osseuse.

1.8. Le magnésium et le métabolisme osseux

Une carence prolongée en Mg^{2+}, condition fréquente dans les pays industrialisés, induit des troubles du métabolisme phosphocalcique et amène à une hypocalcémie chez l'homme et la plupart des espèces animales. Le métabolisme et l'action de la vitamine D peuvent être également perturbés lors de déficit magnésique. L'hypocalcémie dépendante du Mg^{2+} ne peut être corrigée de façon durable que par l'apport de Mg^{2+}, l'apport du Ca^{2+} et de la vitamine D étant inefficace (Rude RK 1998). L'hypocalcémie Mg-dépendante entraîne un défaut de sécrétion de la PTH. De plus le déficit magnésique sévère conduit également à une diminution de la réponse à cette hormone.

En conclusion, l'hypocalcémie Mg-dépendante lors d'un déficit sévère en Mg^{2+} s'explique partiellement par une réponse osseuse non adéquate à la PTH et à la

$1,25(OH)_2D_3$, et par une sécrétion inappropriée des parathyroïdes en réponse à l'hypocalcémie (Anast et Forte 1983).

Toutefois certaines études indiquent qu'un déficit modéré en Mg^{2+}, une condition qui est plus probable d'être retrouvée, altère également le tissu osseux. Ainsi, une diète faible en Mg^{2+} a été identifiée comme un facteur prédisposant à une réduction graduelle de la masse osseuse et au développement de l'ostéoporose chez l'humain (Rude et Gruber 2004). De plus chez le rat et la souris, une diète déficiente en Mg^{2+} entraîne une augmentation du nombre d'ostéoclastes, une diminution du nombre d'ostéoblastes et une perte de la masse osseuse sans que la calcémie et les niveaux de vitamine D ne soient altérés (Rude R *et al* 2005 ; Rude *et al* 2003). Ainsi dans cette situation, la perte osseuse ne proviendrait pas d'une hypocalcémie ou de l'altération des niveaux de PTH et de vitamine D, mais indique un effet direct de la réduction de Mg^{2+} sur l'équilibre du remodelage osseux.

1.9. Le magnésium et sa distribution dans l'organisme

Le Mg^{2+} est le deuxième cation intracellulaire en importance, après le potassium, dont la concentration varie entre 0.4 et 0.9 mM. L'organisme contient environ 1 mole de Mg^{2+} soit environ 24 g, et 60 à 70 % du Mg^{2+} total est situé dans l'os qui constitue une réserve mobilisable. Seulement 1 % du contenu total en Mg^{2+} de l'organisme est présent dans les espaces extracellulaires ; sa concentration plasmatique est de 0,8 mmol · L^{-1} dont 60 % correspond à la forme libre, non liée aux protéines, ni complexée avec des anions (Essig et Amiel 1997).

Le Mg^{2+} est le cofacteur de plus de 300 réactions enzymatiques. Il est également impliqué dans de nombreux processus tels la contraction musculaire, la libération de neurotransmetteurs, la synthèse des protéines, la régulation de l'adényl-

cyclase, d'où l'importance de cet élément dans les fonctions cellulaires. Dans la cellule, le Mg^{2+} est associé à différentes structures et la concentration du Mg^{2+} libre intracellulaire se maintient à un niveau relativement constant, même s'il existe une modification du Mg^{2+} extracellulaire. Ce phénomène résulte d'une perméabilité limitée de la membrane plasmique, de la mise en jeu de systèmes de transport spécifiques qui régulent les entrées et les sorties. Les mécanismes par lequel le Mg^{2+} entre dans la cellule ne sont pas encore parfaitement élucidés. Toutefois, l'efflux de Mg^{2+} semble impliquer principalement un antiport sodium/magnésium. De plus, le Mg^{2+} libre intracellulaire est en équilibre avec le Mg^{2+} lié aux différentes structures cellulaires, constituant un système tampon qui contribue à l'homéostasie du Mg^{2+} intracellulaire.

1.10. L'implication du magnésium dans la prolifération cellulaire

Des études ont indiqué que des mitogènes pouvaient moduler le niveau de Mg^{2+} intracellulaire (Rijkers et Griffioen 1993). La privation de Mg^{2+}, alternativement, entraîne l'inhibition de la synthèse d'ADN et de protéines favorisant de ce fait un arrêt de croissance cellulaire (Sanui et Rubin 1977). Ainsi, le Mg^{2+} semble jouer un rôle important dans la prolifération cellulaire.

1.10.1. Le magnésium intracellulaire en condition de prolifération

Il y a une grande variabilité au niveau de la concentration de Mg^{2+} intracellulaire entre les différents types de cellules allant de 0.4 à 0.9 mM. Toutefois, la grande majorité des données indiquent que les cellules en prolifération ont des

concentrations en Mg^{2+} intracellulaires supérieures aux cellules ne proliférant pas (Rubin H *et al* 1979). En principe, une telle différence serait en accord avec les effets de promotion du Mg^{2+} sur la synthèse protéique et la synthèse d'ADN (Sanui et Rubin 1977; Cameron et Smith 1989).

1.10.2. Le magnésium extracellulaire et la prolifération cellulaire

L'observation que le contenu intracellulaire et la distribution du Mg^{2+} peuvent changer avec la prolifération physiologique ou néoplasique (Rubin H *et al* 1979) a mené beaucoup d'investigateurs à explorer l'influence possible du Mg^{2+} extracellulaire sur ces processus. Entre autre, Rubin et collègues ont étudié la prolifération de fibroblastes d'embryon de poussin et ont constaté que le transport de 2-déoxyglucose, la glycolyse, et la synthèse d'ARN, d'ADN et de protéines exigent la présence de Mg^{2+} dans le milieu de culture (Rubin H *et al* 1979). Une exigence pour le Ca^{2+} a aussi été observée, mais les auteurs ont conclu que les effets métaboliques d'une réduction de Ca^{2+} du milieu ont été finalement atténués par des augmentations du Mg^{2+} extracellulaire (Rubin et Koide 1976). Selon ces études, la hausse du Mg^{2+} extracellulaire stimule la synthèse de protéines et le métabolisme énergétique, permettant le déclenchement de la division cellulaire. De plus, des diminutions modérées du Mg^{2+} extracellulaire (de 0,8 à 0,1 mM) ont empêché la prolifération des fibroblastes d'embryon de poussin, et ce de façon équivalente à la privation de sérum (Rubin et Bowen-Pope 1979). La prolifération des cellules pouvait être reprise en incluant à nouveau le Mg^{2+} dans le milieu de culture (Rubin et Chu 1978).

1.11. Les voies d'entrée cellulaires du magnésium

Le Mg^{2+} est un ion essentiel impliqué dans beaucoup de processus biochimiques et physiologiques. L'homéostasie des niveaux de Mg^{2+} sériques est étroitement réglée et dépend de l'équilibre entre l'absorption intestinale et l'excrétion rénale. Cependant, peu d'information est disponible au sujet des protéines spécifiques assurant l'entrée du Mg^{2+} dans la cellule. Récemment, Goytain et Quamme ont caractérisé plusieurs transporteurs membranaires assurant l'homéostasie du Mg^{2+} au niveau des cellules rénales et épithéliales tels SLC41A1 (Goytain et Quamme 2005b), SLC41A2 (Goytain et Quamme 2005c) et MagT1 (Goytain et Quamme 2005d). De plus, un autre transporteur du magnésium, l'ACDP2 (Goytain et Quamme 2005a), a été identifié au niveau de la membrane plasmique des cellules de plusieurs types tissulaires (rein, cerveau, cœur, intestin, cellules épithéliales). Au niveau rénal, l'expression des gènes SLC41A1, MagT1 et ACDP2 est augmentée en conditions d'hypomagnésie. De plus, il semble que l'expression des gènes MagT1 et ACDP2 soit augmentée en fonction de la réduction du Mg^{2+} extracellulaire.

De plus, les études d'électrophysiologie ont indiqué que certains membres de la famille des TRPM assurent le transport de Mg^{2+} (Nadler *et al* 2001) et que leur ouverture est régulée par la concentration de Mg intracellulaire. Le canal TRPM6 est plus spécifique au Mg^{2+} et assure l'absorption du Mg^{2+} au niveau intestinal et rénal (Nadler *et al* 2001) tandis que le canal TRPM7 est ubiquitaire et semble impliqué dans l'homéostasie du Mg^{2+} cellulaire (Pedersen 2005).

1.11.1. Les canaux TRPM7

Nadler et collaborateurs ont cloné le TRPM7, un membre de la famille des TRP, et ont montré que ce canal engendre un courant Mg^{2+} lorsqu'il est exprimé dans une grande variété de cellules (Nadler *et al* 2001). Au même titre que les autres canaux TRP, l'organisation des TRPM7 rappelle la structure à six régions transmembranaires (S1-6) des canaux potassiques. Le pore permettant le passage des cations est situé entre les segments S5 et S6. Il est à noter que les canaux TRPM7 possèdent dans leurs extrémités carboxy-terminales un domaine kinase qui intervient par un mécanisme d'autophosphorylation dans l'activation du canal et vice versa. De plus, le domaine kinase de TRPM7 phosphoryle d'autres substrats tels l'annexine et la chaîne lourde de la myosine IIA.

Les canaux TRPM7 sont exprimés dans plusieurs tissus (Clapham *et al* 2001). En plus d'être perméables au Ca^{2+} et au Mg^{2+}, les canaux TRPM7 permettent le transport de divers métaux tels le zinc, le manganèse, le cadmium et le cobalt (Monteilh-Zoller *et al* 2003). Une réduction de l'expression de TRPM7 dans les cellules HEK293 entraîne la mort cellulaire (Nadler *et al* 2001). Cependant, la survie de ces cellules peut être assurée en augmentant la concentration du Mg^{2+} dans le milieu de culture (Nadler *et al* 2001). L'importance du canal TRPM7 dans la prolifération cellulaire a aussi été suggérée. Une réduction de l'expression de TRPM7 dans les cellules entraîne un arrêt de croissance après 24 heures. La prolifération de ces cellules pouvait également être retrouvée en augmentant le niveau du Mg^{2+} extracellulaire (Schmitz *et al* 2003). Il a donc été suggéré que le canal TRPM7 soit responsable de l'homéostasie du Mg^{2+} cellulaire.

Des études ont indiqué que des mitogènes pouvaient stimuler la proliferation cellulaire en augmentant le niveau intracellulaire de Mg^{2+} par l'activation des transporteurs membranaires favorisant ainsi un influx de Mg^{2+} extracellulaire (Wolf *et al* 2004; Rubin 2005). À cet égard, le «platelet-derived growth factor» (PDGF) est

un facteur mitogène ubiquiste qui stimule la multiplication et la migration de plusieurs types cellulaires, entre autres des ostéoblastes. Ainsi, le facteur de croissance PDGF pourrait stimuler ou activer les transporteurs membranaires impliqués dans l'influx du Mg^{2+}.

1.12. Le facteur de croissance dérivé des plaquettes (PDGF)

Le PDGF est un polypeptide de 30 kDa, formé de deux chaînes polypeptidiques unies par des ponts disulfure. Ces deux chaînes peuvent être de deux types soit des chaînes A de 125 acides aminés, soit des chaînes B de 160 résidus, présentant 60% d'homologie entre-elles. Selon la nature des chaînes polypeptidiques constitutives, il existe donc 3 isoformes de PDGF: PDGF-AA, PDGF -BB, et PDGF-AB (Fredriksson 2004).

Le PDGF est un facteur mitogène ubiquiste qui exerce sa fonction via son récepteur R-PDGF. Il existe trois types de récepteur : αα, ββ, αβ. La fixation du PDGF sur son récepteur provoque l'activation du domaine protéine kinase du récepteur (Heldin *et al* 1988). Les deux récepteurs associés formant un dimère se phosphorylent réciproquement sur leurs résidus tyrosine. Cette phosphorylation stimule l'activité enzymatique de celui-ci. D'autres résidus seront par la suite phosphorylés, ce qui permet la fixation, par le domaine SH2, de molécules impliquées dans la transduction du signal : Shc, Grb2, Nck, Src, pi3-k, GAP, PTP1D. Par l'intermédiaire de domaines SH2, deux voies de signalisation principales sont activées: la voie des ``mitogen-activated protein kinases`` (MAP kinases), via Ras puis Erk, ce qui amène à l'augmentation de l'expression de l'ARNm de la cycline D. La voie PI3kinase et Akt phosphoryle la cycline D et bloque sa dégradation par ubiquitination tandis qu'elle inhibe également les voies d'apoptose. Le cycle cellulaire est alors activé et la cellule progresse vers la phase S, la mitose et la prolifération

(Bornfeldt 1995). Par la suite le récepteur PDGF est rapidement internalisé et dégradé à l'intérieur de la cellule (Heldin et Westermark 1999).

Le PDGF est un facteur qui stimule la multiplication et la migration de plusieurs types cellulaires tels les fibroblastes, les cellules musculaires lisses, les chondrocytes, les cellules gliales, les ostéoblastes et autres types cellulaires (Mehrotra *et al* 2004). Les facteurs de croissance favorisent notamment la régénération des cellules et des tissus, et certains facteurs de croissance ont été préconisés notamment dans le traitement de l'ostéoporose (Devescovi *et al* 2008). Lors d'une lésion cellulaire *in vivo*, les thrombocytes (ou plaquettes) libèrent de nombreux facteurs de croissance, comme par exemple le PDGF (platelet-derived growth factor), le TGF beta (transforming growth factor beta), les IGF-I et -II (insulin-related growth factor).

L'effet du magnésium et implication des canaux TRPM7 dans les fonctions des cellules ostéoblastiques seront étudies dans les divers modèles ostéoblastiques.

1.13. Modèles cellulaires

Les lignées de cellules issues d'ostéosarcomes humains telles MG-63, SaOS-2, U2OS et les cellules murines MC3T3-E1 sous-clone 4 obtenues de l' ``American Type Culture Collection`` (ATCC) expriment différents marqueurs ostéoblastiques et seront utilisées afin d'étudier la régulation du remodelage osseux.

Les cellules de la lignée MC3T3-E1 sont dérivées de calvaria de souriceaux nouveau-nés et présentent un phénotype normal. *In vitro* les cellules ostéoprogénitrices MC3T3-E1 constituent un excellent modèle de différenciation des précurseurs ostéoblastiques en ostéoblastes matures. Ils synthétisent une matrice de collagène qui se minéralise en cours de différenciation (Wenstrup *et al* 1996).

Les cellules de la lignée MG 63 présentent un phénotype préostéoblastique et proviennent d'un ostéosarcome humain (Sorkin *et al* 2004 ; Billiau *et al* 1977). C'est un modèle largement utilisé pour étudier les fonctions cellulaires. Elles ont cependant un pauvre potentiel de différenciation.

Les cellules de la lignée U2 OS présentent un phénotype ostéoblastique et proviennent également d'un ostéosarcome humain (Sorkin *et al* 2004, Yao et Schaffer 1995). Elles ont comme les MG-63 un pauvre potentiel de différenciation.

Les cellules SaOS sont des ostéoblastes matures et proviennent d'un ostéosarcome humain (Sorkin *el al* 2004, Yao et Schaffer 1995). Ces cellules sont capables de produire des nodules de minéralisation.

1.14. Hypothèses de travail et objectifs

Le maintien de la masse osseuse est intimement relié à un juste équilibre entre la résorption du tissu osseux par les ostéoclastes et la formation d'une nouvelle matrice par les ostéoblastes. Ces deux processus contribuent au remodelage du tissu osseux et impliquent une coordination de l'activité des deux types cellulaires. Puisqu'une diète faible en Mg^{2+} a été identifiée comme un facteur prédisposant à une réduction graduelle de la masse osseuse et à l'ostéoporose, il est important de déterminer les mécanismes à l'origine de cette perte osseuse. Certaines études associent cette condition à une formation osseuse inadéquate due à un nombre réduit d'ostéoblastes. Ainsi, nous croyons que le Mg^{2+} joue un rôle important dans la régulation des activités des ostéoblastes. Un maintien inadéquat du Mg^{2+} en circulation serait à même d'affecter la prolifération, la migration ainsi que la différenciation et la capacité des ostéoblastes à synthétiser et déposer la matrice minérale et ainsi altérer la régulation du remodelage osseux et la formation osseuse. De plus, nous croyons que le canal TRPM7 assure l'homéostasie du Mg^{2+} intracellulaire des ostéoblastes et que la régulation ainsi que la modulation de l'expression de ce canal serait à même de contrôler les activités des ostéoblastes, ainsi que la régulation du remodelage osseux et la formation osseuse.

L'objectif principal de ce projet est de déterminer l'importance du Mg^{2+} extracellulaire et de TRPM7, un canal qui serait responsable d'homéostasie du Mg^{2+} intracellulaire, au niveau des fonctions des ostéoblastes dans le processus du remodelage osseux. Ce projet de recherche a été entrepris selon les quatre objectifs suivants :

1) déterminer l'expression des canaux TRP dans les cellules ostéoblastiques humaines et murines.

L'expression génique des canaux TRPC, TRPM et TRPV sera vérifiée dans les lignées d'ostéoblastes humains (MG-63, SaOS-2, U2OS) et murins MC3T3 par une approche de biologie moléculaire impliquant les réactions ``transcriptase inverse - réaction en chaîne par polymérase`` (RT-PCR).

2) déterminer l'importance des canaux TRPM7 dans l'homéostasie du Mg^{2+} intracellulaire et sur la prolifération des cellules ostéoblastiques.

Dans un premier temps, nous allons déterminer dans les cellules ostéoblastiques (MG-63, SaOS-2, U2OS) si des canaux sont activés lors d'un déficit extracellulaire en Ca^{2+} et/ou Mg^{2+} en mesurant l'influx calcique et du Mg^{2+}. Par la suite, nous tenterons d'établir un lien entre la réduction du Ca^{2+} et/ou du Mg^{2+} extracellulaire et l'activation des canaux TRPM7 afin de préserver l'homéostasie du Ca^{2+} et du Mg^{2+} intracellulaire. Dans un deuxième temps nous évaluerons si l'expression des canaux TRPM7 est augmentée afin de compenser un déficit en Ca^{2+} et/ou en Mg^{2+} extracellulaire. Finalement, nous déterminerons l'influence d'un déficit extracellulaire en Ca^{2+} et/ou Mg^{2+} et l'implication des canaux TRPM7 dans la prolifération des cellules ostéoblastiques.

3) évaluer l'importance du Mg^{2+} et des canaux TRPM7 au niveau de la prolifération et la migration basale des ostéoblastes et induites par le PDGF.

Récemment une étude a indiqué que les canaux TRPM7 contribuent au remodelage du cytosquelette cellulaire en agissant sur la dynamique des filaments d'actomyosine (ClarK *et al* 2006), ce qui influence l'adhésion et le mouvement des cellules neuronales (Su *et al* 2006). De plus, le PDGF est reconnu comme un stimulateur de la prolifération et la migration des ostéoblastes (Mehrotra M et al 2004). Pour cela, nous évaluerons l'importance du Mg^{2+} et des canaux TRPM7 sur la prolifération, l'adhésion et la migration des ostéoblastes en conditions basale et induite par le PDGF.

4) étudier l'implication du Mg^{2+} et des canaux TRPM7 dans la différenciation des ostéoblastes et la minéralisation de la matrice extracellulaire.

Dans un premier temps, nous étudierons l'effet de la réduction du Mg^{2+} extracellulaire sur la différenciation des cellules ostéoblastiques (MC3T3–E1) et nous évaluerons l'expression génique des canaux TRPM7 par RT-PCR en fonction de la différenciation des cellules. Dans un deuxième temps, nous étudierons l'effet de la réduction des canaux TRPM7 sur la différenciation cellulaire. Plusieurs marqueurs de la différenciation ostéoblastique (activité phosphatase alcaline, expression de l'ostéocalcine et potentiel de minéralisation) seront évalués pour des cellules maintenues dans des milieux de culture normale ou faible en Mg^{2+}, ainsi que pour des cellules dont l'expression de TRPM7 sera réduite par l'interférence à l'ARN.

CHAPITRE II

2.1. Article 1 : Expression des canaux ioniques TRP dans les ostéoblastes humains et murins.

Résumé

Le maintien de la masse osseuse dépend en grande partie de la prolifération des cellules ostéoblastiques, et subséquemment de leur différenciation et de la sécrétion de protéines formant la matrice osseuse. Tout récemment est apparu la superfamille des canaux ioniques les ''transient receptor potential'' (TRP). Les TRP jouent un rôle important dans le contrôle des activités cellulaires et sont exprimés dans plusieurs types cellulaires, pourtant peu d'information est disponible sur leur expression et leurs fonctions dans les cellules ostéoblastiques. La présente étude visait à déterminer l'expression et certaines fonctions des canaux ioniques TRP dans plusieurs modèles de cellules ostéoblastiques. Nos résultats montrent que les ostéoblastes humains MG-63, SaOS et U2 OS expriment les canaux TRPC1, TRPC3, TRPC4 et TRPC6, et que les ostéoblastes murins MC3T3 expriment les canaux TRPC2, TRPC4, TRPC6 et TRPC7. De plus les ostéoblastes humains et murins expriment les canaux TRPM4, TRPM6, TRPM7, TRPM8, TRPV2 et TRPV4. Le canal TRPM1 est exprimé seulement dans les ostéoblastes de souris et le canal TRPM3 dans les ostéoblastes humains. Une stratégie d'interférence à l'ARN ciblant TRPC1 prévient l'influx calcique généré selon un mécanisme d'activation de type SOCC ainsi que la prolifération des cellules ostéoblastiques induite par le '' platelet-derived growth factor'' PDGF. De plus, une stratégie d'interférence à l'ARN ciblant TRPM7 réduit la prolifération basale et induite par le PDGF. Cette étude souligne l'expression géniques des canaux TRP et l'importance des canaux TRPC1 et TRPM7 dans la proliferation des cellules ostéoblastiques.

Cet article a été publié en 2009 dans le « Journal of Molecular Membrane Biology ». Il comporte 8 figures. J'ai réalisé l'ensemble des expériences qui ont permis d'obtenir les résultats présentés aux figures 2, 3, 6, 7 et 8 et j'ai participé aux expériences qui ont mené aux résultats apparaissant aux figures 1 et 4. J'ai participé à 80% de la rédaction de l'article sous la supervision de mon directeur de recherche qui a commenté et révisé le document.

Expression of transient receptor potential (TRP) channels in human and murine osteoblast-like cells

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Abstract

The preservation of bone mass relies on adequate proliferation, differentiation, secretion of matrix proteins and rate of apoptosis of the bone-forming osteoblasts. Although growing body of evidence indicates that the transient receptor potential (TRP) channels play important roles in numerous cellular functions, limited information is available about the TRP channels in osteoblasts. Here, we inventoried the gene expression and addressed some roles of the TRP channels in various osteoblast-like cells. The transcripts of canonical TRP (TRPC) channels were revealed for TRPC1, TRPC3, TRPC4 and TRPC6 in human MG-63, SaOS and U2 OS osteoblasts while transcripts for TRPC2, TRPC4, TRPC6 and TRPC7 were observed in the murine MC3T3 osteoblasts. PCR products were shown for the melastatin-related TRP (TRPM) channels TRPM4, TRPM6, TRPM7 and TRPM8 in all cell lines. The TRPM1 was specifically expressed by murine MC3T3 cells while the TRPM3 transcripts were revealed solely in human osteoblast-like cells. Transcripts for TRPV2 and TRPV4 were shown in osteoblastic cells. By interfering RNA approaches, the TRPC1 channels in osteoblasts were shown to be responsible for the capacitative calcium entry (CCE) and for the stimulation of cell proliferation by platelet-derived growth factor. On the other hand, interfering RNA-mediated abrogation of the expression of TRPM7, known as calcium and magnesium channels, resulted in the reduction of both basal and growth factor-stimulated osteoblastic cell

proliferation. Our results provide the first complete reference for the gene expression of TRP channels in osteoblasts and point to their importance in cell proliferation.

Introduction

The bone is a dynamic tissue that is continuously being remodelled following two coordinated processes. The osteoclasts are constantly breaking down old bone (known as the resorption process) as the osteoblasts are replacing it with new tissue (termed the bone formation process). The osteoblastic cells ensure bone formation and tissue mineralization through the secretion of bone matrix components (type I collagen and noncollagenous proteins) and also provide factors essential for the differentiation of osteoclasts. By regulating osteoclast differentiation, the osteoblasts not only play a central role in bone formation, but also in the regulation of bone resorption [1]. Therefore, the maintenance of bone remodelling equilibrium relies on the adequate proliferation, migration, differentiation, secretion of matrix proteins and apoptosis rate of the osteoblastic cells.

The cloning and characterization of members of the transient receptor potential (TRP) cation channel family have exploded during recent years, leading to a plethora of data on the roles of TRP channels in a variety of tissues and species (for a review [2]). Nevertheless, few studies have investigated the expression of TRP channels and addressed their roles in osteoblastic cells. All TRP channels contain six putative transmembrane domains, which are thought to assemble as homo- or hetero-tetramers to form the active channels. All TRPs are cation channels, although the permeability for different mono- and divalent cations varies greatly between isoforms. They are

activated by a wide range of stimuli including intra- and extracellular messengers, chemical, mechanical and osmotic stress, and some by the filling state of intracellular Ca^{2+} stores. Based on amino acid homologies, the mammalian TRP channel superfamily has been divided into seven families: TRPC, TRPM, TRPV, TRPML, TRPP, TRPA and TRPN. The TRPC ('Canonical') and TRPM ('Melastatin') subfamilies consist of seven and eight different channels, respectively (i.e., TRPC1–TRPC7 and TRPM1–TRPM8). The TRPV ('Vanilloid') subfamily presently comprises six members (TRPV1–TRPV6). The TRPML ('Mucolipin') family comprises three members, and the TRPP ('Polycystin') family three channel-like and five non-channel members, respectively. The most recently proposed subfamily, TRPA ('Ankyrin'), has only one mammalian member, TRPA1, and finally, the TRPN (no mechanoreceptor potential C, or NOMPC) has so far only been detected in *Caenorhabditis elegans*, *Drosophila*, and zebra fish. There has been increasing interest in first three families because of their involvement in several human diseases while last four families are yet insufficiently characterized.

Little information is presently available about the expression of TRP channels in bone cells. TRPC3 has been considered as a candidate for mediating store-operated non-selective cation entry into the rat osteoblast-like ROS 17/2.8 cells [3-5], a Ca^{2+} influx activated by either 1alpha,25-dihydroxy-vitamin D3 (1alpha,25$(OH)_2D_3$) or thapsigargin-induced depletion of Ca^{2+} stores. Recently, we reported the gene expression of numerous TRC channels in human osteoblast-like MG-63 cells and

suggested their involvement in the stimulation of osteoblast proliferation by growth factors [6]. While we observed that TRPC channels play important role in the stimulation of osteoblast proliferation, the exact member(s) of the TRPC family was(were) not identified. Also, we have reported the expression of TRPM6 and TRPM7 in human osteoblast-like cells [7]. We demonstrated that TRPM7 channels are involved in the intracellular homeostasis of magnesium and in the basal osteoblast proliferation. Recently, the expression of TRPV4 channels has been shown in osteoblasts and osteoclasts [8]. A physiologic role of TRPV4 in bone adaptation to mechanical stress was proposed as TRPV4 deficient mice were protected from the bone loss induced by unloading. TRPV5 has been shown in human and murine osteoclasts [9], with predominant localization to the ruffled border membrane. In regard to bone metabolism, TRPV5 deficiency leads to an increase in osteoclast size and number, in which bone resorption is nonfunctional.

In the current study, we have inventoried the expression of TRPC, TRPM and TRPV channels in three well known human osteoblast-like cells, namely MG-63, U2 OS and SaOS, and in the murine MC3T3 osteoblast cell line. In addition, we have addressed the roles of TRPC1 and TRPM7 channels in osteoblast proliferation. The potential involvement of TRP channels in bone metabolism is discussed.

Materials and methods

Cell culture

Osteoblast-like cell lines were from the American Type Culture Collection (ATCC; Rockville, MD, USA). Human osteoblastic cells MG-63 were grown in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (DMEM/F12; Sigma, Oakville, Ontario, Canada). Human cell lines SaOS and U2 OS were maintained in McCoy's 5A medium (Hyclone, Logan, UT, USA). Murine osteoblast cell line MC3T3 was grown in alphaMEM medium (Sigma). All media were supplemented with 10% fetal bovine serum (FBS; Cansera, Etobicoke, Ontario, Canada), 2 mM L-glutamine (Invitrogen, Burlington, Ontario, Canada) and penicillin/streptomycin solution (Invitrogen). Cells were cultured in 5% CO_2 at 37 °C and were harvested once a week with a Trypsin-EDTA solution (Invitrogen).

Reverse-transcription polymerase chain reaction

Total RNA was extracted from cells using TriZol (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) reactions were carried out with Omniscript RT kit (Qiagen, Mississauga, Ontario, Canada) using hexamers. Amplifications by polymerase chain reaction (PCR) were conducted with *Taq* PCR core kit (Qiagen) using specific primer sets for human and murine TRP channels (Table 1). Each primer was complementary to distinct exons according to GenBank sequences to ensure the specific amplification of transcripts. Briefly, PCR

amplifications were carried out for 30-40 cycles according to incubation of 1 min at 94°C, 30 sec at 58°C and 1 min at 72°C. Amplification products were resolved by electrophoresis on 2% agarose gel with ethidium bromide revelation.

Measurements of intracellular calcium

Cells were cultured in 4-wells Labtek dishes (Nalge Nunc, Naperville, IL, USA) for 5 days in supplemented media. Cells were then transferred to HEPES-buffered saline solution (mM: 121 NaCl, 5.4 KCl, 0.8 Mg_2SO_4, 25 HEPES, 1.8 $CaCl_2$ and 6.0 $NaHCO_3$ at pH 7.3) and loaded with 2 μM Fluo-3 AM (Invitrogen) with an equivalent volume of 20% Pluronic F127 (Invitrogen) for 45 min at 37°C in the dark. Thereafter the cells were washed with HEPES-buffered saline solution and the loaded dye was allowed to deesterify for 45 min at room temperature in the dark. Following transfer to a Ca^{2+}-free solution (HEPES-buffered saline solution without $CaCl_2$), additions were made in an open chamber configuration at room temperature. The cells were examined with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300) with an Apochromatic 40X N.A. 1.0 objective lens. Fluorescence was excited by an argon laser at 488nm and emissions were collected with a 515 filter. The release of Ca^{2+} from endoplasmic reticulum was induced by the addition of 5μM thapsigargin (Sigma), an inhibitor of endoplasmic reticulum Ca^{2+} pumps. Following addition of Ca^{2+} to the buffer (final concentration of 1.8 mM), a store-operated influx could be obtained. Inhibition of store-operated channels was accomplished by the addition of 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethy]-1H-imidazole

hydrochloride (SKF96365; Sigma) before the addition of Ca^{2+} to the media. Data were analyzed with the Laser Sharp 2.1T, Time Course 1.0 software. The fluorescence of 30-40 cells per field was analyzed. The individual fluorescence intensities of treated cells were compared to the fluorescence intensity before the stimulation and were used to obtain a mean fluorescence ratio for each experiment. The area under the curve was computed using GraphPad Prism version 4 (GraphPad Software, San Diego California, US).

Cell proliferation

The cells were cultured for 3 days in 96-well plates (Sarstedt, Montréal, Québec, Canada) with supplemented media and proliferation was determined by the microtiter tetrazolium (MTT) assay after 48 h of treatment with 10%FBS, 25 ng/ml platelet-derived growth factor BB (PDGF) or culture medium alone. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT, Sigma) was added to the media at a final concentration of 0,5 mg/ml. Four hours later, formazan crystals generated by cellular reduction of the MTT reagent were dissolved in dimethylsulfoxide (Sigma) for 30 min at 37 °C and the absorbance was determined at 575nm. Results are expressed as the proliferation of cells from the MTT activity versus initial MTT values (absorbance values from cells at the initial day of treatment).

Interference with siRNA

Small interfering RNAs obtained from Qiagen (Mississauga, Ontario, Canada) were directed against human TRPM7 (si-hTRPM7, SI00119196), murine TRPM7 (si-mTRPM7, SI02694727), human TRPC3 (si-hTRPC3, SI03078775), human TRPC1 (si-hTRPC1, SI00050449) and a nontargeting control (siRNA(-)). Transfection of the siRNAs was performed using HiPerFect reagent following the manufacturer's instructions. To examine the role of TRPC1 and TRPM7 in cell proliferation, cells plated in 96-well plates (Sarstedt) were transfected for 48 h, and thereafter used for the measurement of intracellular Ca^{2+} or incubated in appropriate conditions for 48 h with the addition of MTT for the last 4 h of incubation.

Statistical analysis

Statistical differences were evaluated by ANOVA with Dunnett's post-test using GraphPad Prism 4. A level of $P<0.05$ was considered significant.

Results

Gene expression of the TRPC channels in osteoblastic cells

The presence of messenger RNAs for TRPC channels was investigated in various human osteoblastic cell models by RT-PCR using specific primers (Table 1). As shown in Fig. 1A, PCR products for the TRPC1 (472 bp), TRPC3 (404 bp with R1 primer in MG-63 and SaOS cells, and 611 bp with R2 primer for U2 OS cells) and TRPC6 (692 bp with F1 primer in MG-63 and SaOS cells, and 824 bp with F2 primer for U2 OS cells) were obtained with the total RNA from human osteoblast-like MG-63, SaOS and U2 OS cells. Interestingly RT-PCR for the TRPC4 led to the amplification of three major bands of 1200, 900 and 700 bp in MG-63 and SaOS cells suggesting the expression of transcripts for splice variants while no PCR product was revealed with the RNA from U2 OS. The gene expression of TRPC5 and TRPC7 was not revealed in human osteoblastic cells using total or messenger RNA, and TRPC2 is considered as a pseudogene in human [10]. In addition, we investigated the expression of TRPC channels in the murine osteoblastic MC3T3 cells. The gene expression of TRPC2, TRPC4, TRPC6 and TRPC7 was revealed in murine MC3T3 cells with the observation of PCR amplification products of 890, 599, 687 and 525 bp respectively (Fig. 1B). The expression of TRPC1, TRPC3, and TRPC5 was not observed with total or messenger RNA.

Gene expression of the TRPM channels in osteoblastic cells

The presence of messenger RNAs for TRPM channels was investigated by RT-PCR in various osteoblastic cell lines. Specific PCR products were obtained with the RNA from human osteoblast-like MG-63, U2OS and SaOS cells indicating the gene expression of TRPM3 (395 bp), TRPM4 (541 bp), TRPM6 (441 bp), TRPM7 (975 bp) and TRPM8 (539 bp) (Fig. 2A). Gene expression of TRPM1, TRPM2 and TRPM5 was not revealed using total or messenger RNA. As shown in Fig. 2B, the gene expression of TRPM1, TRPM4, TRPM6, TRPM7 and TRPM8 was revealed in the murine osteoblastic MC3T3 cells by the observation of PCR amplification products of 279, 952, 269, 814 and 525 bp respectively. In contrast to human osteoblastic cells, the expression of TRPM3 was not observed. The gene expression of TRPM2 and TRPM5 was not revealed using total or messenger RNA.

Gene expression of the TRPV channels in osteoblastic cells

The gene expression of TRPV channels was investigated in human and murine osteoblastic cells. Transcripts for the TRPV2 were revealed in the human osteoblast-like cells with PCR products of 433 bp (Fig. 3A) and in the murine cells with PCR product of 522 bp (Fig. 3B). Also, the gene expression of TRPV4 channels were shown in human osteoblast-like SaOS and U2 OS cells with PCR products of 866 and 686 bp respectively (Fig. 3A) suggesting two splice variants while no PCR product was observed with the RNA of MG-63 cells. In addition, the presence of transcripts

for TRPV4 was revealed in the murine MC3T3 cells with PCR products of 403 bp (Fig. 3B).

Involvement of TRPC channels in the capacitative calcium entry and the proliferation of osteoblastic cells.

Since members of the TRPC channel family have been associated with the induction of CCE following depletion of Ca^{2+} from intracellular stores in human osteoblastic MG-63 cells [6], we investigated the effect a known TRPC channel inhibitor SKF96365 on the induction of CCE in the murine MC3T3 cells, upon thapsigargine (Tg)-induced endoplasmic reticulum Ca^{2+} depletion by performing Fluo-3 fluorescence measurements of intracellular Ca^{2+}. In the absence of external Ca^{2+}, 5 μM Tg induced a transient increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in MC3T3 cells (Fig. 4), which corresponds to the classical depletion of intracellular Ca^{2+} stores. When extracellular Ca^{2+} was reintroduced, a rapid rise in $[Ca^{2+}]_i$ was evoked, which is typical of CCE activation. In contrast, the cells showed no response to the addition of Ca^{2+} in the buffer without prior stimulation with Tg (data not shown) suggesting no significant Ca^{2+} influx following the addition of calcium under basal condition. Following Ca^{2+} store depletion with 5 μM Tg in the absence of extracellular Ca^{2+}, the addition of extracellular Ca^{2+} to the incubation media of cells pre-incubated with the known TRPC inhibitors SKF96365 resulted in the observation of minimal CCE (reduction by 94.3 ± 8.3% in MC3T3 cells; Dunnett's $P<0.01$) (Fig. 4). Similar inhibition by SKF96365 was obtained for the CCE activated by platelet-derived growth factor (PDGF) and serum (data not shown). No significant difference

was noticed for the percentage of cells that responded to Tg in the presence of channel blocker. Similar results were obtained with U2 OS and SaOS cells (data not shown). Altogether, these results indicate the presence of functional TRPCs in osteoblastic cells which are responsible for the establishment of CCE upon depletion of intracellular Ca^{2+} stores.

In order to address the role of channels of the TRPC family in the functions of osteoblastic cells, we investigated the effects of the SKF96365 on the proliferation of osteoblastic cells. As shown in Fig. 5, cell proliferation promoted by 10% FBS was reduced by SKF96365 in a dose-dependent manner in all human osteoblast-like cell lines. Similar results were observed for the murine MC3T3 cells (data not shown). Also, PDGF-stimulated proliferation was inhibited by SKF96365 in a dose-dependent manner in MG-63 and U2 OS cells, while the inhibitor was without effect on PDGF-unresponsive SaOS cells indicating that this inhibitor acted specifically on the stimulated cell proliferation. It should be noted that although SKF96365 has been used as TRP channel blockers, it also blocks chloride channels, ICRAC and second-messenger-activated non-selective currents [11]. Nevertheless as our results suggest that one or numerous channels of the TRPC family are involved in the proliferation of osteoblastic cells, we further investigated the involvement of specific TRPC channels in osteoblast functions.

Involvement of TRPC1 and TRPC3 in the CCE and osteoblast proliferation

Of the TRPC family, TRPC1 and TRPC3 have been often considered as responsible for CCE. As murine MC3T3 cells did not express these channels, we therefore investigated the involvement of TRPC1 and TRPC3 in the CCE of human MG-63 cells. As TRPC3 channels have been shown to be responsible of the CCE induced upon 1alpha,25$(OH)_2D_3$- or thapsigargin-induced depletion of intracellular Ca^{2+} stores in the rat osteoblast-like ROS17/2.8 cells [3], we investigated its involvement in our conditions. As shown in Fig 6A, treatment of MG-63 cells with siRNA against TRPC3 led to a specific reduction of the expression of TRPC3. However, intracellular Ca^{2+} measurements showed that CCE was still observed upon depletion of intracellular Ca^{2+} stores by PDGF in the cells where TRPC3 expression has been abrogated (Fig. 6B). Moreover, transfection with siRNA against TRPC3 was without effect on the stimulation of MC3T3 proliferation by PDGF (1.49 ± 0.03 vs 1.45 ± 0.05 for mock and si-TRPC3 conditions respectively). As our results showed that TRPC3 channels were not responsible of CCE under our condition, we speculated that TRPC1 channels may account for the establishment of CCE. Under conditions where the expression of TRPC1 is reduced by 80% using interfering RNA (Fig. 7 A), the CCE is nearly abolished (Fig. 7B). Moreover, PDGF did not stimulate cell proliferation in the osteoblasts transfected with the siRNA against TRPC1 while the basal cell proliferation was not affected (Fig. 7C).

Involvement of TRPM7 channels in the proliferation of osteoblastic cells.

One of the most characterized members of the TRPM channel family is TRPM7, a calcium and magnesium channel, which has been involved in cell proliferation [12;13]. In order to evaluate the role of TRPM7 in osteoblastic cells, we determined the effects of RNA interference against TRPM7 on cell proliferation. The relative expression of TRPM7 evaluated by RT-PCR 2 days after the transfection showed a reduction of 80% (Fig. 8A). Abrogation of TRPM7 expression led to reduction of basal cell proliferation in the U2 OS and MG-63 and cells (Fig. 8B). Moreover, the stimulation of cell proliferation by PDGF was abolished by the siRNA against TRPM7. Similar results were obtained with the MC3T3 cells (data not shown). The silencing of TRPM7 expression had no effect on the depletion of intracellular Ca^{2+} stores and the activation of CCE (data not shown). Therefore, our results indicate that TRPM7 channels are involved in the basal and stimulated proliferation of osteoblastic cells.

Discussion

TRPC channels in osteoblastic cells

On the basis of sequence homology and functional similarities, members of the mammalian TRPC family have been divided into four subfamilies: TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5, where TRPC2 is a pseudogene in human. It should be noted that the expression of at least one member of each subfamily was shown in human osteoblastic cell lines, namely TRPC1, TRPC4 and TRPC3/6 with the exception of TRPC4 in U2 OS cells (Table 2). On the other hand, the pattern of expression of TRPCs in murine MC3T3 cells was remarkably distinct from human osteoblast-like cells. Although the expression of TRPC4 and TRPC6 was also observed as in human osteoblastic cells, no transcript for TRPC1 was revealed. Instead, the specific expression of TRPC2 and TRPC7 was found in murine cells. Differences in the pattern of TRPC expression could be related to species. On the other hand, the level of osteoblastic maturity may influence the pattern of TRPC expression since MG-63, U2OS are rather preosteoblasts and SaOS mature osteoblasts, whereas MC3T3 cells were maintained in the current study as osteoprogenitors. Further investigation of TRPC expression along osteoblastic differentiation would be of interest.

The existence of numerous splice variants has increased the functional multiplicity of TRPC channels. All members of the TRPC family are believed to share a common

topology [14;15]. The cytoplasmic N- and C-termini are separated by six predicted transmembrane domains (TM1–TM6), in addition of a putative pore region between TM5 and TM6. TRPs oligomerized as hetero or homotetramers to form ion channels. The N-terminus of TRPCs is composed of three to four ankyrin repeats, a predicted coiled coil region and a putative caveolin binding region. The cytoplasmic C-terminus includes the TRP signature motif (EWKFAR), a highly conserved proline rich motif, the CIRB (*c*almodulin/IP_3 *r*eceptor *b*inding) region and a predicted coiled-coil region. An extended C-terminus containing a PDZ binding motif is unique to TRPC4 and TRPC5. In view of the TRPCs expressed in osteoblastic cells, short (**NM_003305**) and long (TRPC3a, **AY865574**) variants for human TRPC3 have been reported with an extension of 73 amino acids in the N-terminus segment of the protein which has been proposed to influence the activation property of the channel from receptor-operated to store-operated channel [16]. However, the identification of latter variants for TRPC3 was not possible with the primer set that we used. Splice variants for hTRPC6 (called delta316-431, **AJ271067** and delta377-413, **AJ271068**) lacking the first out of six hydrophobic regions predicted to span the plasma membrane have been reported, although the protein expression and the functional meaning are still unknown. However, the identification of latter variants for TRPC6 was not possible with the primer set that we used. Numerous splice variants have been reported for human TRPC4. The three major bands revealed by RT-PCR on human osteoblast-like MG-63 and SaOS cells may correspond to transcripts

previously reported as TRPC4 and variants α, β, ε, δ (PCR product of 1210 bp; **NM_016179**, **AF421358**, **AF421359**, **AF421360**, **AF063824**), TRPC4ζ (PCR product of 873 bp; **AF421361**) and TRPC4ζ (PCR product of 691 bp; **AF421362**). Further investigations are warranted in order to identify the exact variant(s) of TRPC4 expressed in osteoblastic cells. Although the primer set for murine TRPC2 did not permit to reveal splice variants, isoforms with a shorter N-terminus (**AF111107**, **AF230802** and **AF230803**) and with differences in the sequence for the C-terminus segment (**AY465411**) have been reported. Moreover, splice variants leading to shorter N-terminus segments have been identified for murine TRPC6 (**AF057748**) and C-terminus domain of TRPC4 (**U50921** and **AF019663**).

There is evidence that TRPC channels can be activated, following phospholipase C activation, by DAG (as receptor-operated Ca^{2+} channel, ROCC), by direct interaction with the Ca^{2+} release channels of the endoplasmatic reticulum, the IP_3 receptor and the ryanodine receptors, by translocation to the plasma membrane via an exocytotic process and by store-operated mechanisms (as store-operated Ca^{2+} channel, SOCC) [17;18]. Although some debates persist about the exact mode of activation, our results indicate that the human TRPC1 account for capacitative calcium current (CCE) in osteoblastic cells. In contrast, TRPC3 has been shown in rat osteoblast-like ROS 17/2.8 cells to mediate capacitative calcium entry induced by thapsigargine and $1,25(OH)_2D_3$ [3]. It should be noted that our results indicated differences on the expression of TRPC channels with the expression of TRPC1 and TRPC3 in human

and solely TRPC2 expression in murine osteoblast cells. Therefore similar functions to TRPC1 and TRPC3 may be ensured by the murine TRPC2. With the exception of TRPC3, the expression of other TRPC channels is currently unknown in rat osteoblast cells and differences in the expression of TRPC members between human and rat osteoblasts may underlie opposite results. Moreover, the prevailing mode of channel operation, ROCC or SOCC, has been shown to depend on the differential degree of TRPC3 expression levels which influence the final tetraoligomeric composition [19]. Again, the levels of TRPC3 expression and of other TRPC members are unknown in rat osteoblasts. On the other hand, human and murine TRPC6 as well as murine TRPC7 may account for DAG-activated cation entry. It is generally accepted that CCE is triggered in order to ensure proper Ca^{2+} refilling of intracellular stores [17] and it is thought to be an essential component of the long-term responses of the cell, including proliferation. In accordance, our results indicate that TRPC1 channels are involved in the stimulated osteoblast proliferation by PDGF. Interestingly, TRPC1 has been shown to form stretch-activated cation channels [20] which may be related to the increase in bone formation upon mechanical loading.

TRPM channels in osteoblastic cells

On the basis of the proposed four groups (TRPM1/3, TRPM2/8, TRPM4/5 and TRPM6/7), again at least one member of each TRPM subfamily was shown to be expressed in all our osteoblastic cell lines, namely TRPM3, TRPM4, TRPM6/7 and TRPM8 for human cells, and TRPM1, TRPM4, TRPM6/7 and TRPM8 in murine

cells (Table 3). The main difference was the expression of TRPM1 by murine osteoblastic cells compared to the expression of TRPM3 by human cells. Again, the differences in the pattern of TRPM expression could be related to species or the level of osteoblastic maturity and further investigation is warranted.

TRPM proteins lack ankyrin repeats in the N-terminus but contain the TRP domain in the C-terminus [21]. The N-terminal part of TRPM proteins is considerably longer than the corresponding regions in TRPC, contains a large, 700 amino acids 'TRPM homology region'. The C-terminus of TRPM proteins is considerably longer than the corresponding region of other TRP and some members of the TRPM family (TRPM2, TRPM6 and TRPM7) contain enzyme domains. These TRPM proteins are thus called "chanzymes." Among all 9 variants identified to date for TRPM3 and in view of our PCR product of 395 bp obtained for TRPM3 in human osteoblast-like cells, splice variants 1 (**NM_020952**), 2 (**NM_024971**), 3 (**NM_206944**), 4 (**NM_206945**), 7 (**NM_206948**) and 9 (**NM_001007471**) are possible. On the other hand variants 5 (**NM_206946**), 6 (**NM_206947**) and 8 (**NM_001007470**) are excluded since PCR product would be of 470 bp. Former variants show deletion of exons 10 to 12. Variants B and C of TRPM4 are likely according to PCR product of 541 bp whereas splice variant A for TRPM4 are excluded since PCR product would be of 184 bp. Expression of TRPM6 was revealed by RT-PCR (amplicon of 441 bp). Since M-6-Kinase variants were reported, we performed RT-PCR with specific primers for M-6-

kinase 1 to 3 (**AY333286**, **AY333287**, **AY333288** respectively). No PCR product was obtained (data not shown) confirming the expression of TRPM6 channel.

TRPM channels exhibit highly varying permeability to Ca^{2+} and Mg^{2+}, from Ca^{2+}-impermeable (TRPM4 and 5) to highly Ca^{2+} and Mg^{2+} permeable (TRPM6 and 7) [22]. In view of the TRPM channels shown to be expressed in osteoblastic cells, little is known about the functional properties and cellular functions of TRPM1. This channel was identified and named melastatin because of the absence of TRPM1 mRNA in malignant transformed melanoma cell lines, suggesting a tumor suppressor function of the channel protein [23;24]. TRPM3 channels have been shown to be expressed primarily in kidney and, at lesser levels in several tissues such as, in brain, testis, and spinal cord [22;25-27]. TRPM3 channels showed a constitutive Ca^{2+} entry characterized by activation at positive potentials and deactivation at negative potentials, suggesting a voltage-dependent gating of TRPM3 [28]. Depletion of intracellular Ca^{2+} stores further augmented TRPM3-mediated Ca^{2+} entry [27]. Moreover, the Ca^{2+} entry in TRPM3-expressing HEK293 cells increased during treatment with hypotonic extracellular solution [26] suggesting volume-regulated activity of TRPM3. Physiological function in the kidney was therefore attributed to Ca^{2+} homeostasis. TRPM4 forms calcium-activated sodium channels impermeable for calcium and have been suggested to mediate depolarization of the plasma membrane [22;25]. Furthermore, Nilius et al [29] reported that phosphatidylinositol 4,5-biphosphate (PIP2) may constitute a physiologically important brake on TRPM4

activity since activation of the phospholipase C (PLC)-coupled receptors potently inhibited currents through TRPM4. Cheng et al [30] reported that in insulin-secreting cells of the rat pancreatic beta-cell line INS-1, a dominant negative TRPM4 construct result in significantly decreased insulin secretion in response to a glucose stimulus.

The TRPM7 channels have been associated with cell proliferation and survival [31], exhibiting spontaneously activated divalent cation (Ca^{2+}, Mg^{2+}, and other trace metals) entry regulated by the cytosolic Mg^{2+} and ATP levels [32]. Expression of TRPM7 has been reported in neurons [33], lymphocytes [34], cardiac myocytes [35], vascular smooth muscle cells [13], and is thought to be widely expressed. TRPM7 channels have been associated with cell viability [31], cellular Mg homeostasis [36], anoxic neuronal cell death [33], intestinal pacemaking activity [37], cell proliferation of retinoblastoma cells [12], vascular smooth muscle cell proliferation and response to fluid flow [13]. Our previous results have indicated that TRPM7 is involved in the intracellular Mg homeostatis of osteoblastic cells as well as in basal cell proliferation [7]. In the current study, we report that TRPM7 channels are also involved in PDGF stimulated osteoblast proliferation.

The cDNA of TRPM8 was first isolated from prostate cancer cells, and the function of TRPM8 was initially linked to progression of cancer cells [38]. TRPM8 expression was also found in a subset of cold-responsive dorsal root ganglia neurons and in neurons from trigeminal ganglia as a cold receptor of the body [39]. Heterologously expressed TRPM8 channels showed that TRPM8 channels are permeable to Ca^{2+} and

are activated by cooling cells to <24 °C and by application of cooling agents such as menthol, icilin and eucalyptol. However, such thermosensation in osteoblastic cells may not be the main function of TRPM8 and further studies are needed in order to address its physiological roles in bone metabolism.

Expression of TRPV channels in osteoblastic cells.

Our results indicate that only TRPV2 and TRPV4 are expressed in osteoblastic cells (Table 4). In accordance with our current results, van der Eerden et al. [9] have reported the expression of TRPV5 in osteoclasts but not in primary cultures of human and murine osteoblasts. TRPV2, also called VRL-1 and growth factor-regulated channel (GRC), is a weakly Ca^{2+}-selective cation channel which is a member of the so-called thermo-TRPs (TRPV1-4) that are activated by heating in heterologous expression systems and presumably appeared to function in thermosensation of sensory neurons (for a review [40]). Whereas TRPV1 is activated by temperature higher than 43°C, TRPV3 responds to a lower temperature range of 32–40°C [41]. TRPV2 on the other hand was shown to respond to a noxious temperature of >52°C [42], thereby its physiological role as such remains unsubstantiated. These channels are also expressed in cell and tissues with no known thermoregulatory homeostasis such as lung alveolar macrophages, epithelial cells of various human tissues such as kidney, stomach, intestine and prostate [43], in neutrophil granulocytes [44], in skeletal, vascular and cardiac muscle cells [45;46], in human hepatoblastoma (HepG2) cells [47]. In osteoblastic cells TRPV2 main role may not be thermosensation.

Moreover TRPV1 and TRPV2 have been shown to be not essential to normal heat responses [48]. TRPV2 channels are emerging as multifunctional receptors which respond to growth factors [49;50], is proposed to play a role in proinflammatory degranulation events in mast cells [51] and in mechanosensation as a stretch-activated channel in vascular smooth muscle [45]. Latter function in mechanosensation is more likely in osteoblastic cells since beneficial effects of mechanical loading on bone structure are known [52]. Recently, the expression of TRPV4 channels has been shown in osteoblasts and osteoclasts [8]. A physiologic role of TRPV4 in bone adaptation to mechanical stress was proposed as TRPV4 deficient mice were protected from the bone loss induced by unloading.

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Table 1. Sequences of the primers used for PCR amplification.

Gene	Sequences	Accession number
hTRPC1	F: 5'-GTTTCATGATTTTGCTGATCGGA-3'	NM_003304
	R: 5'-AGCACAATCACAACCACGACATT-3'	
hTRPC3	F: 5'-TCCAAGACGCTGAACGTCAAC-3'	NM_003305
	R1: 5'-CACTTGCAGAAATAGTCGTGCG-3'	
	R2*: 5'-TTGCATGGAGAGCTTCCGATAG-3'	
hTRPC4	F: 5'-AGAAGTCGTCGGAGCTGTTGAG-3'	NM_016179
	R: 5'-CCAGAGATATTTGCAGAGGTCCC-3'	
hTRPC6	F1: 5'-AGAAGGGGAGAAGGTTAGCTAATCG-3'	NM_004621
	F2*: 5'-GACTATCTGCTCATGGACTCGGAG-3'	
	R: 5'-CAGTTCATTGCTAAGTTCTAAAGCCG-3'	
mTRPC2	F: 5'-CAGGACTATGGCTTCGAGTTGC-3'	NM_011644
	R: 5'-GAACCGGATCATGTCGTCAATC-3'	
mTRPC4	F: 5'-ACAATACAGTCAGCCAACGCG-3'	NM_016984
	R: 5'-GGGCTTAAGTCATAGTCTATGCTCGAG-3'	
mTRPC6	F: 5'-AGTTCATTGAAAACATCGGCTACG-3'	NM_013838
	R: 5'-CCAGCTTTGGCTCTAACGACAG-3'	
mTRPC7	F: 5'-GCTTACGGCAACAGTCTATCGC-3'	NM_012035
	R: 5'-GTGAGACGTTGTGCAGCGTTAC-3'	
hTRPM3	F: 5'-GAGGGGTTAACACAGGTGTTATTCG-3'	NM_206946
	R: 5'-CTCGAAGGTACTCCAAAACAATCG-3'	
hTRPM4	F: 5'-GTTCATAGTTGACTCCACAGATCCG-3'	NM_017636
	R: 5'-GTTGATGAGGGTGTCTCTATTCCG-3'	
hTRPM6	F: 5'-TCACTGGACCTATGAGTACACTCGG-3'	NM_017662
	R: 5'-GACGCTGATGTAATCAACATCACG-3'	
hTRPM7	F: 5'-TGCACCTATACTAGGAAACGTTTTCG-3'	NM_017672
	R: 5'-CATGATAAAAGGCATAAAACTTTCGC-3'	
hTRPM8	F: 5'-GTATATACGTCTGTCCTGCGACACG-3'	NM_024080
	R: 5'-CCATAGTTGGAATCTTGAATAGTGCG-3'	
mTRPM1	F: 5'-CGAGGAGCTTCTGTTTGAGG-3'	NM_018752
	R: 5'-GCTTCCGTGCTCTTGAAATC-3'	

mTRPM4	F: 5'-AAGCCAAGTGTCTCCGAGCTGC-3'	**NM_175130**
	R: 5'-TCAGCAGCTTCCGCTCTGCTTC-3'	
mTRPM6	F: 5'-AAGGCGGTCATTGCTAGTATCC-3'	**NM_153417**
	R: 5'-TGGGTACAAGAATGCGACAC-3'	
mTRPM7	F: 5'-ATGACGATGGAGGATAGTG-3'	**NM_021450**
	R: 5'-TTGTCGGGAGAGTGGAGTC-3'	
mTRPM8	F: 5'-AAGAGGACAGAAGCAGCAGG-3'	**NM_134252**
	R: 5'-AATGATACGAGGCCACAGCC-3'	
hTRPV2	F: 5'-CAAGGGACTTGCTTTTATTTCGG-3'	**NM_016113**
	R: 5'-CAGAAGCCAGGTCATACAGCG-3'	
hTRPV4	F: 5'-ACACCAAGTTTGTTACCAAGATGTACG-3'	**NM_021625**
	R: 5'-CGATCATGAAGAGCAAGTAGACGAG-3'	
mTRPV2	F: 5'-ATGGAGCGAATGTTCACATCC-3'	**NM_011706**
	R: 5'-TGATTTCCAGGACCGAGTTC-3'	
mTRPV4	F: 5'-AAGATGTACGACCTGCTGC-3'	**NM_022017**
	R: 5'-TAGGAGACCACGTTGATGTAGAAGG-3'	
hGAPDH	F: 5'-GAGTCAACGGATTTGGTCGTATTG-3'	**NM_002046**
	R: 5'-GCTGTAGCCAAATTCGTTGTC-3'	
mGAPDH	F: 5'-GGAGATTGTTGCCATCAACGA-3'	**NM_008084**
	R: 5'-TGGGAGTTGCTGTTGAAGTCG -3'	

Table 2: Gene expression of the TRPC channels in human and murine osteoblastic cells.

	Human osteoblast-like cells			Murine osteoblastic cells
TRPC channels	MG-63	SaOS	U2 OS	MC3T3-E1
TRPC1	+	+	+	-
TRPC2	NA	NA	NA	+
TRPC3	+	+	+	-
TRPC4	+	+	-	+
TRPC6	+	+	+	+
TRPC7	-	-	-	+

Table 3: Gene expression of the TRPM channels in human and murine osteoblastic cells

	Human osteoblast-like cells			Murine osteoblastic cells
TRPM channels	MG-63	SaOS	U2 OS	MC3T3-E1
TRPM1	-	-	-	+
TRPM3	+	+	+	-
TRPM4	+	+	+	+
TRPM6	+	+	+	+
TRPM7	+	+	+	+
TRPM8	+	+	+	+

Table 4. Gene expression of the TRPV channels in human and murine osteoblastic cells

	Human osteoblast-like cells			Murine osteoblastic cells
TRPV channels	MG-63	SaOS	U2 OS	MC3T3-E1
TRPV2	+	+	+	+
TRPV4	-	+	+	+

References

[1] Mackie E. 2003. Osteoblasts: novel roles in orchestration of skeletal architecture. Int J Biochem Cell Biol 35: 1301-1305.

[2] Pedersen SF, Owsianik G, Nilius B. 2005. TRP channels: an overview. Cell Calcium 38: 233-252.

[3] Baldi C, Vazquez G, Calvo JC, Boland R. 2003. TRPC3-like protein is involved in the capacitative cation entry induced by 1alpha,25-dihydroxy-vitamin D3 in ROS 17/2.8 osteoblastic cells. J Cell Biochem 90: 197-205.

[4] Santillan G, Baldi C, Katz S, Vazquez G, Boland R. 2004. Evidence that TRPC3 is a molecular component of the 1alpha,25(OH)2D3-activated capacitative calcium entry (CCE) in muscle and osteoblast cells. J Steroid Biochem Mol Biol 89-90: 291-295.

[5] Santillan G, Katz S, Buitrago C, Boland R. 2004. 1alpha,25(OH)2D3 induces capacitative calcium entry involving a TRPC3 protein in skeletal muscle and osteoblastic cells. Biol Res 37: 647-651.

[6] Labelle D, Jumarie C, Moreau R. 2007. Capacitative calcium entry and proliferation of human osteoblast-like MG-63 cells. Cell Prolif 40: 866-884.

[7] Abed E, Moreau R. 2007. Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation. Cell Prolif 40: 849-865.

[8] Mizoguchi F, Mizuno A, Hayata T, Nakashima K, Heller S, Ushida T, Sokabe M, Miyasaka N, Suzuki M, Ezura Y, Noda M. 2008. Transient receptor potential vanilloid 4 deficiency suppresses unloading-induced bone loss. J Cell Physiol 216: 47-53.

[9] van der Eerden BC, Hoenderop JG, de Vries TJ, Schoenmaker T, Buurman CJ, Uitterlinden AG, Pols HA, Bindels RJ, van Leeuwen JP. 2005. The epithelial Ca^{2+} channel TRPV5 is essential for proper osteoclastic bone resorption. Proc Natl Acad Sci USA 102: 17507-17512.

[10] Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C. 1995. TRPC1, a human homolog of a Drosophila store-operated channel. Proc Natl Acad Sci USA 92: 9652-9656.

[11] Franzius D, Hoth M, Penner R. 1994. Non-specific effects of calcium entry antagonists in mast cells. Pflugers Arch 428: 433-438.

[12] Hanano T, Hara Y, Shi J, Morita H, Umebayashi C, Mori E, Sumimoto H, Ito Y, Mori Y, Inoue R. 2004. Involvement of TRPM7 in cell growth as a

spontaneously activated Ca2+ entry pathway in human retinoblastoma cells. J Pharmacol Sci 95: 403-419.

[13] Beech DJ. 2005. Emerging functions of 10 types of TRP cationic channel in vascular smooth muscle. Clin Exp Pharmacol Physiol 32: 597-603.

[14] Vannier B, Zhu X, Brown D, Birnbaumer L. 1998. The membrane topology of human transient receptor potential 3 as inferred from glycosylation-scanning mutagenesis and epitope immunocytochemistry. J Biol Chem 273: 8675-8679.

[15] Vazquez G, Wedel BJ, Aziz O, Trebak M, Putney JW Jr. 2004. The mammalian TRPC cation channels. Biochim Biophys Acta 1742: 21-36.

[16] Yildirim E, Kawasaki BT, Birnbaumer L. 2005. Molecular cloning of TRPC3a, an N-terminally extended, store-operated variant of the human C3 transient receptor potential channel. Proc Natl Acad Sci USA 102: 3307-3311.

[17] Parekh AB, Putney JW Jr. 2005. Store-operated calcium channels. Physiol Rev 85: 757-810.

[18] Freichel M, Vennekens R, Olausson J, Stolz S, Philipp SE, Weissgerber P, Flockerzi V. 2005. Functional role of TRPC proteins in native systems: implications from knockout and knock-down studies. J Physiol 567: 59-66.

[19] Trebak M, Vazquez G, Bird GS, Putney JW. 2003. The TRPC3/6/7 subfamily of cation channels. Cell Calcium 33: 451-461.

[20] Maroto R, Raso A, Wood TG, Kurosky A, Martinac B, Hamill OP. 2005. TRPC1 forms the stretch-activated cation channel in vertebrate cells. Nat Cell Biol 7: 179-185.

[21] Huang CL. 2004. The transient receptor potential superfamily of ion channels. J Am Soc Nephrol 15: 1690-1699.

[22] Kraft R, Harteneck C. 2005. The mammalian melastatin-related transient receptor potential cation channels: an overview. Pflugers Arch 451: 204-211.

[23] Duncan LM, Deeds J, Hunter J, Shao J, Holmgren LM, Woolf EA, Tepper RI, Shyjan AW. 1998. Down-regulation of the novel gene melastatin correlates with potential for melanoma metastasis. Cancer Res 58: 1515-1520.

[24] Deeds J, Cronin F, Duncan LM. 2000. Patterns of melastatin mRNA expression in melanocytic tumors. Hum Pathol 31: 1346-1356.

[25] Harteneck C. 2005. Function and pharmacology of TRPM cation channels. Naunyn Schmiedebergs Arch Pharmacol 371: 307-314.

[26] Grimm C, Kraft R, Sauerbruch S, Schultz G, Harteneck C. 2003. Molecular and functional characterization of the melastatin-related cation channel TRPM3. J Biol Chem 278: 21493-21501.

[27] Lee N, Chen J, Sun L, Wu S, Gray KR, Rich A, Huang M, Lin JH, Feder JN, Janovitz EB, Levesque PC, Blanar MA. 2003. Expression and characterization of human transient receptor potential melastatin 3 (hTRPM3). J Biol Chem 278: 20890-20897.

[28] Grimm C, Kraft R, Schultz G, Harteneck C. 2005. Activation of the melastatin-related cation channel TRPM3 by D-erythro-sphingosine. Mol Pharmacol 67: 798-805.

[29] Nilius B, Mahieu F, Prenen J, Janssens A, Owsianik G, Vennekens R, Voets T. 2006 The Ca^{2+}-activated cation channel TRPM4 is regulated by phosphatidylinositol 4,5-biphosphate. EMBO J 25: 467-478.

[30] Cheng H, Beck A, Launay P, Gross SA, Stokes AJ, Kinet JP, Fleig A, Penner R. 2007. TRPM4 controls insulin secretion in pancreatic beta-cells. Cell Calcium 41: 51-61.

[31] Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurosaki T, Kinet JP, Penner R, Scharenberg AM, Fleig A. 2001. LTRPC7 is

a Mg.ATP-regulated divalent cation channel required for cell viability. Nature 411: 590-595.

[32] Fleig A, Penner R. 2004. The TRPM ion channel subfamily: molecular, biophysical and functional features. Trends Pharmacol Sci 25: 633-639.

[33] Aarts M, Iihara K, Wei WL, Xiong ZG, Arundine M, Cerwinski W, MacDonald JF, Tymianski M. 2003. A key role for TRPM7 channels in anoxic neuronal death. Cell 115: 863-877.

[34] Perraud AL, Knowles HM, Schmitz C. 2004. Novel aspects of signaling and ion-homeostasis regulation in immunocytes. The TRPM ion channels and their potential role in modulating the immune response. Mol Immunol 41: 657-673.

[35] Gwanyanya A, Amuzescu B, Zakharov SI, Macianskiene R, Sipido KR, Bolotina VM, Vereecke J, Mubagwa K. 2004. Magnesium-inhibited, TRPM6/7-like channel in cardiac myocytes: permeation of divalent cations and pH-mediated regulation. J Physiol 559: 761-776.

[36] Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, Kurosaki T, Fleig A, Scharenberg AM. 2003. Regulation of vertebrate cellular Mg2+ homeostasis by TRPM7. Cell 114: 191-200.

[37] Kim BJ, Lim HH, Yang DK, Jun JY, Chang IY, Park CS, So I, Stanfield PR, Kim KW. 2005. Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. Gastroenterology 129: 1504-1517.

[38] Tsavaler L, Shapero MH, Morkowski S, Laus R. 2001. Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. Cancer Res 61: 3760-3769.

[39] McKemy DD, Neuhausser WM, Julius D. 2002. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature 416: 52-58.

[40] Ramsey IS, Delling M, Clapham DE. 2006. An introduction to TRP channels. Annu Rev Physiol 68: 619-647.

[41] Desai BN, Clapham DE. 2005. TRP channels and mice deficient in TRP channels. Pflugers Arch 451: 11-18.

[42] Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D. 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398: 436-441.

[43] Kowase T, Nakazato Y, Yoko O, Morikawa A, Kojima I. 2002. Immunohistochemical localization of growth factor-regulated channel (GRC) in human tissues. Endocr J 49: 349-355.

[44] Heiner I, Eisfeld J, Halaszovich CR, Wehage E, Jungling E, Zitt C, Luckhoff A. 2003. Expression profile of the transient receptor potential (TRP) family in neutrophil granulocytes: evidence for currents through long TRP channel 2 induced by ADP-ribose and NAD. Biochem. J 371: 1045-1053.

[45] Muraki K, Iwata Y, Katanosaka Y, Ito T, Ohya S, Shigekawa M, Imaizumi Y. 2003. TRPV2 is a component of osmotically sensitive cation channels in murine aortic myocytes. Circ Res 93: 829-838.

[46] Iwata Y, Katanosaka Y, Arai Y, Komamura K, Miyatake K, Shigekawa M. 2003. A novel mechanism of myocyte degeneration involving the Ca2+-permeable growth factor-regulated channel. J Cell Biol 161: 957-967.

[47] Vriens J, Janssens A, Prenen J, Nilius B, Wondergem R. 2004. TRPV channels and modulation by hepatocyte growth factor/scatter factor in human hepatoblastoma (HepG2) cells. Cell Calcium 36: 19-28.

[48] Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM. 2004. Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci 24: 6410-6415.

[49] Kanzaki M, Zhang YQ, Mashima H, Li L, Shibata H, Kojima I. 1999. Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. Nat Cell Biol 1: 165-170.

[50] Boels K, Glassmeier G, Herrmann D, Riedel IB, Hampe W, Kojima I, Schwarz JR, Schaller HC. 2001. The neuropeptide head activator induces activation and translocation of the growth-factor-regulated Ca(2+)-permeable channel GRC. J Cell Sci 114: 3599-3606.

[51] Stokes AJ, Shimoda LM, Koblan-Huberson M, Adra CN, Turner H. 2004. A TRPV2-PKA signaling module for transduction of physical stimuli in mast cells. J Exp Med 200: 137-147.

[52] Pavalko FM, Norvell SM, Burr DB, Turner CH, Duncan RL, Bidwell JP. 2003. A Model for mechanotransduction in bone cells: The load-bearing mechanosomes. J Cell Biochem 88: 104-112.

Legend to the figures

Fig. 1. **Gene expression of the TRPC channels in osteoblast-like cells.** Complementary DNA synthesised with total RNA isolated from human (A) or murine (B) cells was used for PCR amplifications using specific primers for each human TRPC (hTRPC) and murine TRPC (mTRPC) channels. Representative data are shown from RNA isolations of at least three independent cultures. Left lane: 100 bp ladder.

Fig 2. **Gene expression of the TRPM channels in osteoblast-like cells.** Total RNA isolated from human (A) or murine (B) cells was used to synthesize complementary DNA for PCR amplifications using specific primers for each human TRPM (hTRPM) or murine TRPM (mTRPM) channels. Representative data are shown from RNA isolations of at least three independent cultures. Left lane: 100 bp ladder.

Fig. 3. **Gene expression of the TRPV channels in osteoblast-like cells.** Complementary DNA synthesised with total RNA isolated from human (A) or murine (B) cells was used for PCR amplifications using specific primers for human TRPV (hTRPV) or murine TRPV (mTRPV) channels. Representative data are shown from RNA isolations of at least three independent cultures. Left lane: 100 bp ladder.

Fig. 4. **Induction of capacitative calcium entry in osteoblastic MC3T3 cells.** Fluo3-loaded MC3T3 cells without or with pre-incubation with 30 μM SKF96365 for 15 min were treated with 5 μM thapsigargine (Tg) in Ca^{2+}-free HEPES-buffered saline solution. After Tg-mediated intracellular Ca^{2+} release, Ca^{2+} was added (right arrow, final concentration of 1.8 mM) to the buffer alone. Each response is expressed as the mean ± SEM of the relative fluorescence intensity from at least 3 experiments with cumulating analysis of between 30 and 40 cells per field.

Fig. 5. **Effect of the TRPC inhibitor SKF96365 on osteoblastic cell proliferation.** MG-63 (A), SaOS (B) and U2 OS (C) osteoblasts were cultured for 48h in DMEM-F12 supplemented with 10% FBS or 25 ng/ml PDGF and increasing concentrations of SKF96365. Cell proliferation was determined after 48 h by the MTT assay and data are the mean ± SEM of the cell proliferation compared to control condition in culture medium from 3 to 6 independent experiments. Statistical analyses were performed by ANOVA with Dunnett's post-test ($P<0.001$ from 2 μM SKF96365 for MG-63 with either PDGF or FBS; $P<0.001$ from 5 μM SKF96365 for SaOS with FBS; $P<0.001$ from 2 μM SKF96365 for U2 OS with either PDGF or FBS)

Fig. 6. **Effect of reducing TRPC3 expression on the capacitative calcium entry induced by PDGF.** MG-63 cells were transfected with specific siRNAs against human TRPC3 (si-hTRPC3) or with nontargeting control siRNA

(Mock). A) The expression of human TRPC1 and TRPC3 was determined after 48 h by RT-PCR and normalized according to the expression of GAPDH for 3 experiments. Student t test: *P<0.05 compared to Mock condition. B) Fluo3-loaded MG-63 cells transfected for 48 h with si-hTRPC3 or nontargeting control siRNA (Mock) were treated with 25 ng/ml PDGF in Ca^{2+}-free HEPES-buffered saline solution. After PDGF-mediated intracellular Ca^{2+} release, Ca^{2+} was added (right arrow, final concentration of 1.8 mM) to the buffer alone. Each response is expressed as the mean ± SEM of the relative fluorescence intensity from at least 3 experiments with cumulating analysis of between 30 and 40 cells per field.

Fig. 7. **Effect of reducing TRPC1 expression on the capacitative calcium entry and cell proliferation induced by PDGF.** MG-63 cells were transfected with specific siRNAs against human TRPC1 (si-hTRPC1) or with nontargeting control siRNA (Mock). A) The expression of human TRPC1 was determined after 48 h by RT-PCR and normalized according to the expression of GAPDH for 3 experiments. Student t test: *P<0.05 compared to Mock condition. B) Fluo3-loaded MG-63 cells transfected for 48 h with si-hTRPC1 or nontargeting control siRNA (Mock) were treated with 25 ng/ml PDGF in Ca^{2+}-free HEPES-buffered saline solution. After PDGF-mediated intracellular Ca^{2+} release, Ca^{2+} was added (right arrow, final concentration of 1.8 mM) to the buffer alone. Each response is expressed as the mean ± SEM of the

relative fluorescence intensity from at least 3 experiments with cumulating analysis of between 30 and 40 cells per field. Statistical analyses were performed by ANOVA with Dunnett's post-test $P<0.001$. C) The cell proliferation was determined by MTT assays on 48-h transfected cells with si-hTRPC1, nontrageting control (Mock) or in culture medium (CTL) following subsequent treatments of 48 h without or with 25 ng/ml PDGF. Data are the mean ± SEM of the cell proliferation compared to CTL without PDGF from 3 to 6 independent experiments. Student t test: ***$P<0.001$ compared to CTL without PDGF, $^{£££}P<0.001$ compared to Mock with PDGF.

Fig 8. **Effect of reducing TRPM7 expression on the proliferation of osteoblasts.** Cells were transfected with specific siRNAs against human TRPM7 (si-hTRPM7) or with nontargeting control siRNA (Mock). A) The expression of human TRPM7 in U2 OS cells was determined after 48 h by RT-PCR and normalized according to the expression of GAPDH for 3 independent experiments. Student t test: ***$P<0.001$ compared to Mock. B) Cell proliferation was determined after 48 h for control cells in the culture medium alone (CTL) or for cells following 48-h transfection with nontargeting control siRNA (Mock) or with si-hTRPM7 in the absence or the presence of 25 ng/ml PDGF. Results of initial MTT values (absorbance values from cells at the initial day of treatment) are also shown. Data are expressed as the mean ± SEM of the cell proliferation compared to CTL of 3 independent experiments. Student t test analysis for U2 OS cells: *$P<0.05$ compared to Mock condition,

$^{ff}P<0.001$ compared to Mock condition with PDGF; MG-63 cells: ***$P<0.001$ compared to Mock condition, $^{fff}P<0.001$ compared to Mock condition with PDGF.

Fig. 1. Gene expression of the TRPC channels in osteoblast-like cells

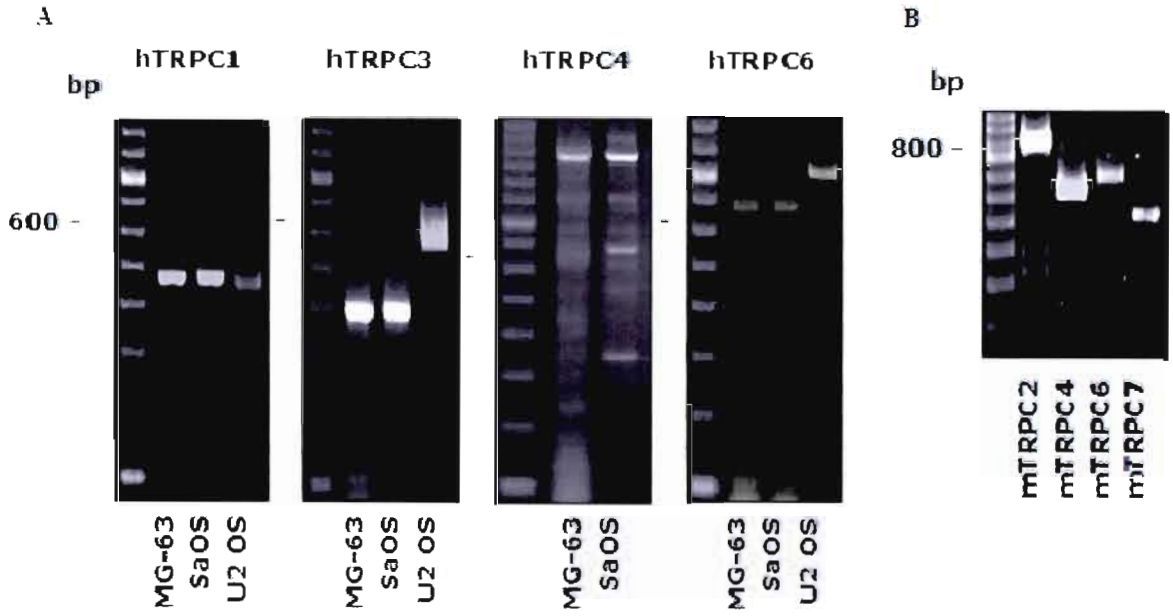


Fig. 2. Gene expression of the TRPM channels in osteoblast-like cells.

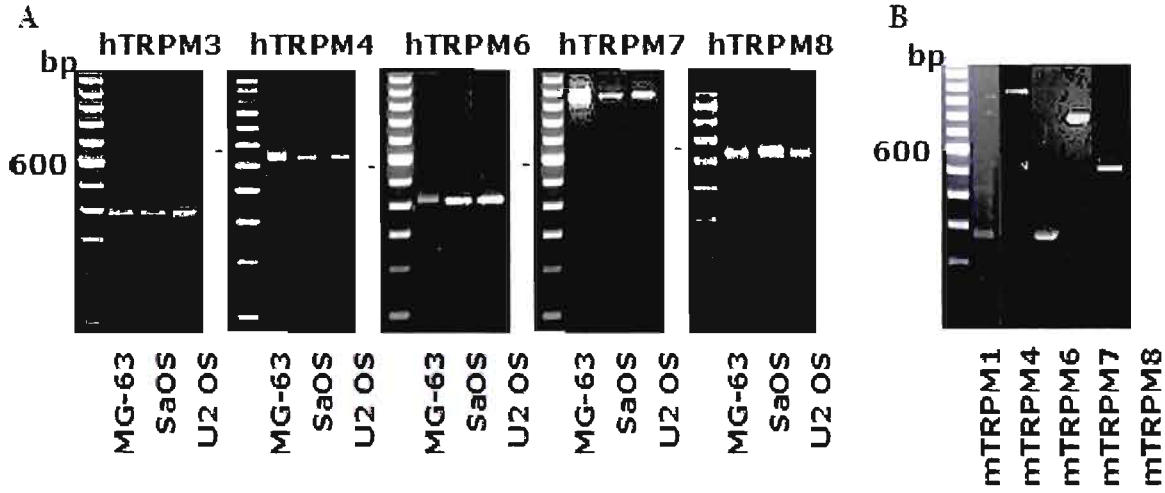


Fig. 3. Gene expression of the TRPV channels in osteoblast-like cells

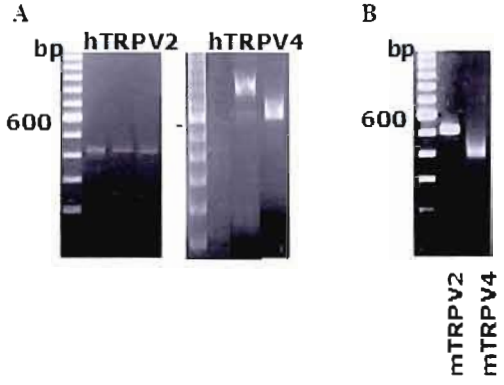


Fig. 4. Induction of capacitative calcium entry in osteoblastic cells

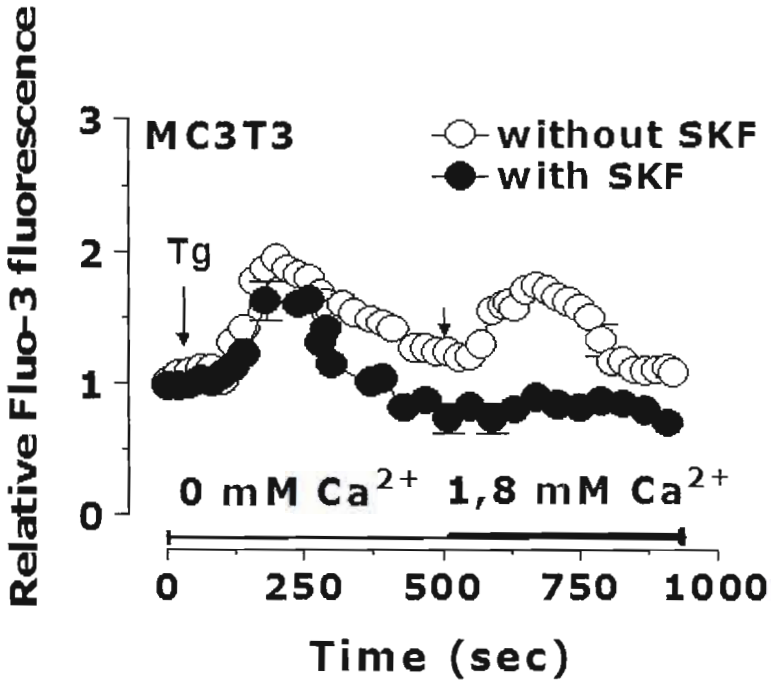


Fig. 5. Effect of the TRPC inhibitor SKF96365 on osteoblastic cell proliferation

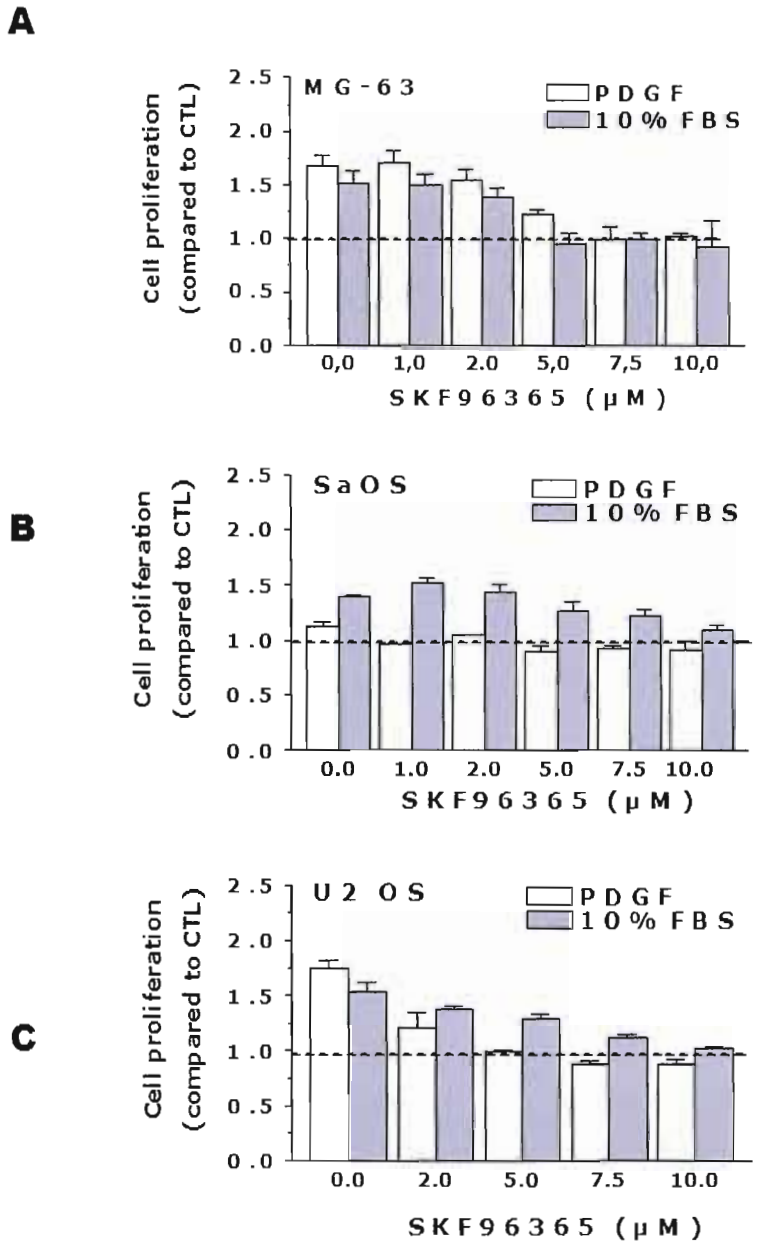


Fig. 6. Effect of reducing TRPC3 expression on the capacitative calcium entry induced by PDGF.

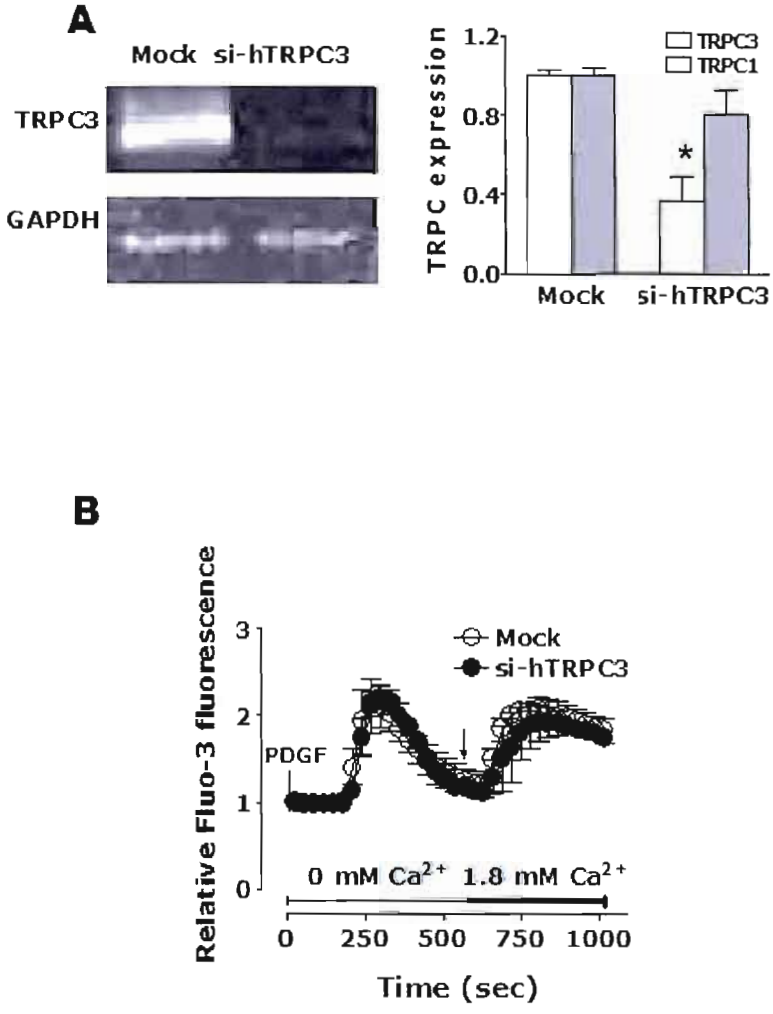


Fig. 7. Effect of reducing TRPC1 expression on the capacitative calcium entry and cell proliferation induced by PDGF.

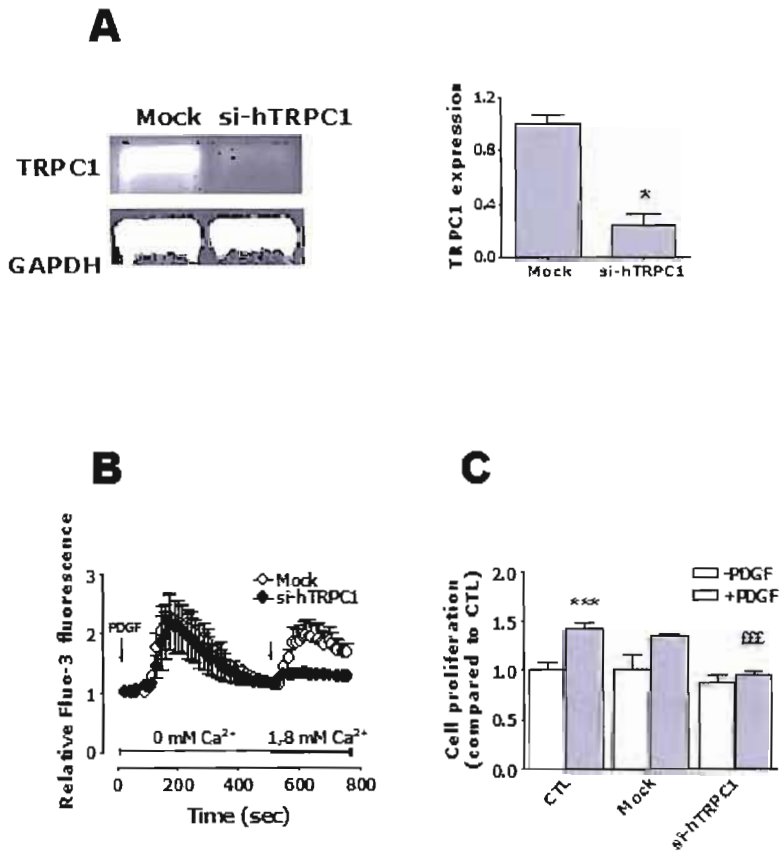
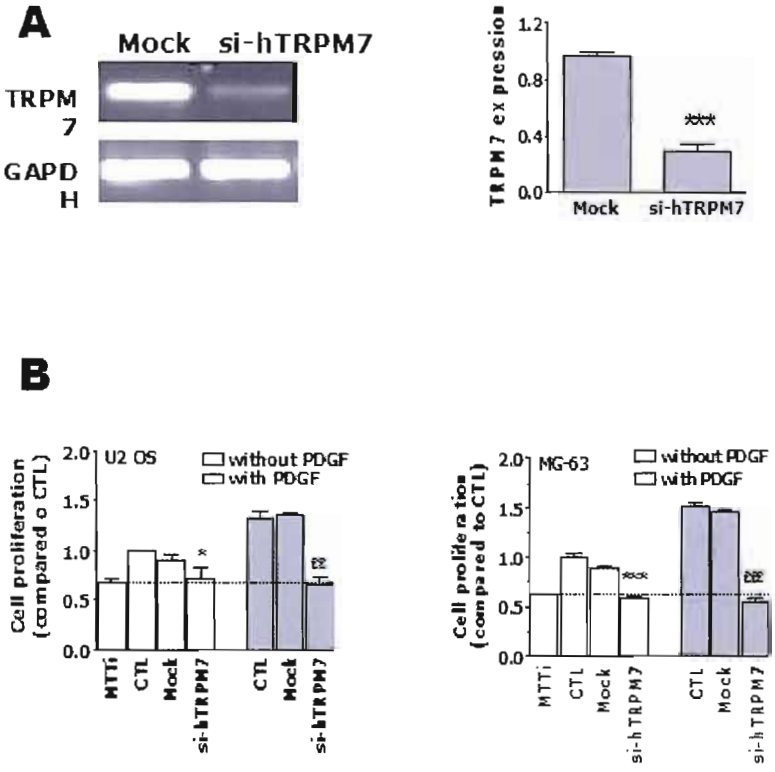


Fig 8. Effect of reducing TRPM7 expression on the proliferation of osteoblasts



2.2. Article 2 : Rôle des cations (Calcium/magnesium) et implication des canaux ioniques "melastatin-related transient receptor potentiel 7" (TRPM7) dans la prolifération des cellules ostéoblastiques humaines.

Le maintien de la masse osseuse au cours du remodelage constant de l'os est assuré par un équilibre entre la résorption du tissu osseux effectuée par les ostéoclastes et sa formation assurée par les ostéoblastes. Cet équilibre dépend en grande partie de la prolifération et de la migration des cellules ostéoblastiques, et subséquemment de leur différenciation et de la sécrétion des protéines formant la matrice osseuse. La présente étude visait à déterminer l'importance des canaux ioniques (calcium/magnesium) "melastatin-related transient receptor potential 6 et 7" (TRPM6, TRPM7) dans la prolifération des cellules ostéoblastiques. Nous avons démontré l'expression génique des canaux TRPM6 et TRPM7 dans les modèles d'ostéoblastes humains MG-63, SaOS et U2OS. La prolifération cellulaire est diminuée dans les conditions de culture faible en calcium ou en magnésium. L'expression de TRPM7 est augmentée dans les conditions de culture ou le calcium et le magnésium extracellulaires sont réduits tandis que l'expression de TRPM6 est demeurée inchangée suggérant l'implication de TRPM7 dans l'homéostasie du calcium et du magnésium intracellulaire. De plus, une réduction du magnésium extracellulaire favorise l'activation des canaux TRPM7 se traduisant par des influx calcique et de magnésium. Des mesures de synthèse d'ADN et de prolifération cellulaire ont indiqué qu'une réduction de magnésium et de calcium dans le milieu de culture diminue la prolifération des ostéoblastes sans affecter leur phénotype. De plus, une stratégie d'interférence à l'ARN ciblant TRPM7 prévient l'induction des influx calcique et de magnésium et réduit la prolifération des cellules ostéoblastiques humaines. En conclusion, nos résultats indiquent que la disponibilité du magnésium extracellulaire influence l'expression et l'activation du canal TRPM7 *in vitro* et qu'une réduction de l'expression de TRPM7 entraîne une diminution de la prolifération cellulaire des ostéoblastes. Cette étude souligne l'importance du calcium et du magnésium au niveau de la prolifération des cellules ostéoblastiques et suggère un rôle de TRPM7 dans cette fonction cellulaire.

Cet article a été publié en 2007 dans le journal « Cell proliferation ». J'ai réalisé toutes les expériences dans ce manuscrit et rédigé le document sous la supervision de mon directeur de recherche qui a commenté et révisé le document.

Importance of melastatin-like transient receptor potential 7 (TRPM7) and cations (magnesium, calcium) in human osteoblast-like cell proliferation

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Running title: Role of TRPM7 in osteoblastic cell proliferation

Keywords: TRPM7 channels; osteoblasts; cell proliferation; magnesium; calcium

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Abstract

The bone tissue in adult is continuously being remodelled and bone mass is maintained constant by the balance between osteoclastic bone resorption and osteoblastic bone formation. Adequate osteoblastic proliferation is mandatory for both appropriate formation and regulation of resorption, and thereby the maintenance of bone remodelling equilibrium. We investigated the roles of melastatin-like transient receptor potential 6 and 7 (TRPM6, TRPM7), two calcium (Ca^{2+}) and magnesium (Mg^{2+}) conducting channels, in the proliferation of human osteoblastic cells. Genetic expression of TRPM6 and TRPM7 was shown in human osteoblast-like MG-63, SaOS and U2 OS cells. The reduction of extracellular Mg^{2+} or Ca^{2+} led to a decrease of cell proliferation. Concomitant reduction of both ions further accentuated the reduction of cell proliferation. The expression of TRPM7 channels was increased under conditions of reduced extracellular Mg^{2+} and Ca^{2+} levels whereas the expression of TRPM6 was not modified suggesting compensatory mechanisms afforded by TRPM7 in order to maintain the intracellular ion homeostasis. Pre-incubation of cells in reduced extracellular Mg^{2+} conditions led to the activation of Ca^{2+} and Mg^{2+} influx. The reduction of TRPM7 expression by specific siRNA prevented latter influx and inhibited cell proliferation. Our results indicate that extracellular Mg^{2+} and Ca^{2+} deficiency reduces the proliferation of human osteoblastic cells. The expression and activity of TRPM7 is modulated by extracellular Mg^{2+} and Ca^{2+} availability indicating that TRPM7 channels are involved in intracellular ion homeostasis and proliferation of osteoblastic cells.

Introduction

Bone is a dynamic tissue that is continuously remodeled at coordinated rates. Under normal condition, special cells called osteoclasts are transiently breaking down old bone (resorption process) at numerous sites as other cells known as osteoblasts are replacing it thereafter to synthesis new tissue (bone formation). Osteoblastic cells ensure bone formation and mineralization through secretion of bone matrix components (type I collagen and noncollagenous proteins) and also provide factors essential for the differentiation of osteoclasts such as macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-kappaB ligand (RANKL). By regulating osteoclast differentiation, osteoblasts not only play a central role in bone formation, but also in the regulation of bone resorption (Mackie 2003). In this regard, adequate osteoblastic proliferation, differentiation, secretory functions or rate of apoptosis is mandatory for both adequate formation and resorption processes, and thereby bone remodelling equilibrium maintenance. Clinical and histomorphometric studies have demonstrated that aging is associated with decreased bone mass and that reduced bone formation is an important pathogenetic factor (Kragstrup *et al.* 1983; Parfitt 1991; Brockstedt *et al.* 1993). In rodent models of aging, studies have indicated a deficit of osteoprogenitor cells in the bone marrow, and a reduction in the number of osteoblasts (Liang *et al.* 1992; Roholl *et al.* 1994; Quarto *et al.* 1995; Bergman *et al.* 1996; Kotev-Emeth *et al.* 2000; Chen 2004). Similar observations were obtained for

human, in addition with a defect in cell proliferation (Pfeilschifter *et al.* 1993; Kato *et al.* 1995; Neidlinger-Wilke *et al.* 1995; Bergman *et al.* 1996; D'Ippolito *et al.* 1999; Martinez *et al.* 1999). Cellular aging and senescence have been reported *in vitro* with human osteoprogenitor cells from bone marrow and osteoblasts (Kassem *et al.* 1997; Stenderup *et al.* 2003; Peterson *et al.* 2004). Therefore with aging the process of coupled bone formation is affected by the reduction of osteoblast proliferation and life span (Chan and Duque 2002). In view of osteoporotic disorders, research regarding osteoblastic cell proliferation could bring new insights on therapeutic approaches.

Calcium influx is implicated in numerous cellular functions such as proliferation, differentiation, secretion and apoptosis (Berridge *et al.* 2000). In bone cells of the osteoblast lineage, Ca^{2+} channels play fundamental roles in cellular responses to external stimuli including both mechanical forces and hormonal signals (Duncan *et al.* 1998; Iqbal and Zaidi 2005). Magnesium is also involved in the regulation of a large number of biochemical reactions and therefore influences important physiological functions such as nucleic acid metabolism, protein synthesis, and energy production (Romani and Scarpa 2000). The "membrane, magnesium mitosis" (MMM) model of proliferation control suggests that upon mitogenic stimuli cells are able to increase their intracellular Mg^{2+} content, likely by activating Mg^{2+} influx, to levels optimum for the initiation of protein synthesis (Wolf *et al.* 2004; Rubin 2005). Therefore,

influx of both extracellular Ca^{2+} and Mg^{2+} for proper intracellular ion homeostasis is likely solicited for cell proliferation.

TRPM is a recently emerging subfamily of the transient receptor potential (TRP) protein, a diverse group of voltage-independent Ca^{2+}-permeable cation channels expressed in mammalian cells (Harteneck *et al.* 2000; Clapham *et al.* 2001; Montell *et al.* 2002; Minke and Cook 2002) that encompasses eight distinct members designated as TRPM1–8. TRPM7 combines structural elements of both an ion channel and a protein kinase (for recent reviews see (Fleig and Penner 2004; Harteneck 2005). Amongst TRPM members TRPM2, TRPM6, and TRPM7 uniquely possess an enzyme domain in their long C-termini, the latter two exhibiting spontaneously activated divalent cation (Ca^{2+}, Mg^{2+}, and other trace metals) entry regulated by the cytosolic Mg^{2+} and ATP levels. Recently TRPM7 channels have been associated with cell proliferation and survival (Nadler *et al.* 2001). TRPM7-deficient DT40 cells can be rescued from their cell growth defect by supplementary extracellular Mg^{2+} (Schmitz *et al.* 2005) suggesting a residual capacity of these cells to maintain intracellular Mg^{2+} homeostasis. Mutations in the TRPM6 gene have been shown in patients suffering from a hereditary form of hypomagnesemia causes by impaired Mg^{2+} reabsorption (Chubanov *et al.* 2004). Life-long dietary Mg^{2+} supplementation of these patients is sufficient to rescue the phenotype of affected human beings. Furthermore, it has been shown that Mg^{2+} deficiency leads to upregulation of TRPM6 in mouse kidneys (Groenestege *et al.* 2006). However,

TRPM7 deficiency cannot be complemented by heterologously expressed TRPM6 (Schmitz *et al.* 2005) suggesting that both channels are not functionally redundant.

In the present study, we investigated potential correlation between the Ca^{2+}- and Mg^{2+}-transporting activities of TRPM7 and the regulation of human osteoblast-like cell proliferation.

Materials and methods

Cell culture

Human osteoblast-like MG-63, SaOS and U2 OS cells were from the American Type Culture Collection (ATCC, Rockville, MD, USA). MG-63 cells were grown in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (DMEM/F12; Sigma, Oakville, Ontario, Canada) and supplemented with 10% fetal bovine serum (FBS; Cansera, Etobicoke, Ontario, Canada), L-glutamine (Invitrogen, Burlington, Ontario, Canada) and penicillin/streptomycin (Invitrogen, Burlington, Ontario, Canada). SaOS and U2 OS cells were cultured in McCoy's 5A medium (Hyclone, Logan, UT, USA) and supplemented as described above. Cells were cultured in 5% CO_2 at 37 °C and were harvested once a week with Trypsin-EDTA solution (Invitrogen, Burlington, Ontario, Canada).

Cell proliferation assays

For proliferation experiments, cells were seeded in 24-or 96-well plates (Sarstedt, Montréal, Québec, Canada) at 2500 cells/cm^2. After 4 days of culture in supplemented media, cells were incubated in Ca- and Mg-free DME/F12 (Sigma, Oakville, Ontario, Canada) medium without serum supplemented with different concentrations of Ca and Mg for 48 h. Cell proliferation was determined by hematocytometer cell counting (24-well plates) or by microtiter tetrazolium (MTT) reduction assay (96-well plates). Briefly for MTT assays, one hour before the end of

the treatment medium was replaced with DMEM/F12 containing 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) (Sigma, Oakville, Ontario, Canada). At the end of the incubation, media was aspired and formazan crystals were dissolved in DMSO. Absorbance was measured at 575 nm and data are expressed as the ratio of absorbance of treated cells vs initial MTT absorbance that correspond to the level of MTT reduced into formazan crystals by cells the initial day of the treatment.

For the evaluation of DNA synthesis, cells were seeded in 96-well plates (Sarstedt, Montréal, Québec, Canada) at 2500 cells/cm^2 and allowed to growth for 4 days in supplemented DMEM/F12. Thereafter, cells were incubated in Ca^{2+}- and Mg^{2+}-free DME/F12 media containing different concentrations of Ca^{2+} and Mg^{2+} or with 25 ng/ml of PDGF-BB (Sigma, Oakville, Ontario, Canada) in DMEM/F12 as positive control. DNA synthesis was determined by BrdU incorporation into cellular DNA using ELISA kit (Roche Diagnostics, Laval, Québec, Canada) following the procedure described by the manufacturer. Briefly, cells were incubated with BrdU for the last 2 h of incubation and thereafter cells were fixed and incubated with nuclease solution. Incorporation of BrdU was determined by incubation with anti-BrdU-POD for 30 min and revealed by peroxidase substrate. Data are expressed as the mean ± SEM of the percentage of DNA synthesis compared to DMEM/F12.

Alkaline Phosphatase activity

Measurement of alkaline phosphatase activity was performed by colorimetric assay of enzyme activity as described previously (Moreau *et al.* 1997). Cell monolayers (24-well plates) were washed three times with PBS buffer (0.1 g/l $CaCl_2$, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 0.1 g/l $MgCl_2.6H_2O$, 8 g/l NaCl, 1.44 g/l Na_2HPO_4, pH 7.4) and then scraped into assay buffer (100 mM glycine, 1mM $MgCl_2$, 1mM $ZnCl_2$, 1% Triton X-100, pH 10.5). Assays were performed in 96-well plates with 75 µl of lysate mixed with 75 µl of freshly prepared colorimetric substrate *para*-nitrophenyl phosphate (Sigma, Oakville, Ontario, Canada) solubilized in the assay buffer. The enzymatic reaction was conducted for 1 h at 37 °C and was stopped by adding 100 µl of NaOH 0.2N. The optical density of the yellow product *para*-nitrophenol was determined spectrophotometrically at 410 nm. Protein concentrations were quantified by MicroBCA protein assay (Pierce, Rockford, IL, USA) using BSA as standard. Alkaline phosphatase activity was then expressed as *para*-nitrophenol produced in nmol/1h/mg of cellular protein.

PCR amplifications

Total RNA from cells was extracted using TriZol (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's instructions. Reverse transcription (RT) reactions were carried out with Omniscript RT kit (Qiagen, Mississauga, Ontario, Canada) using hexamers. The PCR amplifications were conducted with *Taq* PCR

core kit (Qiagen, Mississauga, Ontario, Canada) using specific primer sets for human TRPM7 (sense: 5'-TGCACCTATACTAGGAAACGTTTTCG-3'; antisense: 5'-CATGATAAAAGGCATAAAACTTTCGC-3') and for TRPM6 (sense: 5'-TCACTGGACCTATGAGTACACTCGG-3'; antisense: 5'-GACGCTGATGTAATCAACATCACG -3'). Each primer was designed in distinct exons to ensure specific transcript amplifications. Briefly, amplifications were carried out for 40 cycles according to incubation of 1 min at 94°C, 30 sec at 58°C and 1 min at 72°C. Amplification products were resolved in 2% agarose gel with ethidium bromide revelation.

For Real-time quantitative PCR (RQ-PCR) analysis, RNA were extracted using the Rneasy kit (Qiagen, Mississauga, Ontario, Canada) and cDNA were synthesized with Ominiscript RT kit using hexamers. RQ-PCR were performed with a LightCycler 1.5 system (Roche Diagnostics, Laval, Québec, Canada) using SYBR Premix Ex Taq solution (Takara Bio) and specific primer sets for human TRPM7 (purchased from Qiagen, Mississauga, Ontario, Canada) and normalized to the housekeeping gene beta-2-microglobulin expression (sense: 5'-ATCCAGCGTACTCCAAAGA-3'; antisense: 5'- GACAAGTCTGAATGCTCCAC-3'). Amplifications were performed in LightCycler capillaries (20µl) and data were analyzed with the LightCycler software version 3.5.

Interference with siRNA

Small interfering RNAs directed against human TRPM7 (si-TRPM7(1) and si-TRPM7(2)) and a nontargeting control (siRNA(-)) were obtained from Qiagen (Mississauga, Ontario, Canada). Transfection of the siRNAs was performed using HiPerFect reagent following the manufacturer instructions. Quantifications of transcripts were performed by RQ-PCR 2 days post-transfection to evaluate the TRPM7 expression level. To examine the role of TRPM7 in cell proliferation, cells plated 96-well plates were transfected for 24 h, and thereafter incubated in appropriate conditions for 48 h with the addition of MTT for the last 1 h of incubation.

Measurements of intracellular calcium and magnesium levels

MG-63 cells were cultured in 4-wells Labtek (Nalge Nunc) for 5 days in supplemented media. Cells were then transferred to HEPES-buffered saline solution (HBSS (mM): 121 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 25 HEPES, 1.8 $CaCl_2$ and 6.0 $NaHCO_3$ at pH 7.3) or Ca^{2+}-free and/or Mg^{2+}-free solution (HBSS without Ca^{2+} and/or without Mg^{2+}) and loaded with 2μM Fluo-3 AM or Magnesium Green (Molecular Probes) with an equivalent volume of 20% Pluronic F127 (Molecular Probes) for 45 min at room temperature in the dark. Thereafter cells were washed with corresponding HBSS and the loaded dye was allowed to deesterify for 45 min at room temperature in the dark. Following transfer to a Ca^{2+}-free and Mg^{2+}-free HBSS, additions of Ca^{2+} or Mg^{2+} were made in an open chamber configuration at room temperature. The cells were examined with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300)

with an Apochromatic 40X N.A. 1.0 objective lens. Fluorescence was excited by an argon laser at 488nm and emission was collected with a 515 filter. Data were analyzed with Laser Sharp 2.1T, Time Course 1.0 software.

Statistical analysis

Statistical differences were evaluated using GraphPad Prism3. A level of $P<0.05$ was considered significant.

Results

Expression of TRPM6 and TRPM7 channels in human osteoblast-like cells.

Since some members of the TRPM family, especially TRPM6 and TRPM7, were identified as spontaneously activated Ca^{2+} and Mg^{2+} entry channels suggested to be essential to cell proliferation, we evaluated their expression in human osteoblast-like cell lines. As shown in Fig. 1A, amplicons of expected size (976 bp) corresponding to the presence of messenger RNA for human TRPM7 were revealed by RT-PCR in MG-63, SaOS and U2 OS cells. The expression of TRPM6 was also shown in osteoblastic cell lines (Fig. 1 B, amplicons of 441 bp). As related channel with enzyme domain, the presence of transcripts for TRPM2 was not revealed by RT-PCR (data not shown). The identity of the amplicons was confirmed by restriction enzyme digestion (data not shown).

Influence of reduced extracellular magnesium and calcium on the osteoblast proliferation

Since the expression of TRPM6 and TRPM7 was shown in osteoblastic cells, we determined the importance of extracellular Ca^{2+} and Mg^{2+} in the proliferation of osteoblastic cells. MTT assays were performed on MG-63 cells maintained for 48 h in Ca^{2+}- and Mg^{2+}-free DME/F12 supplemented with various concentrations of both ions. Experiments were performed in the absence of serum in order to exclude the addition of Ca^{2+} and Mg^{2+} from the FBS. In serum-free condition, MG-63 cells showed a basal cell proliferation (2.12 ± 0.21 relative to the initial MTT activity after

48 h). As shown in Fig. 2A, concentrations of Mg^{2+} below 0.8 mM reduced cell proliferation at all concentrations of Ca^{2+}. No significant difference of cell proliferation was observed for concentrations of Mg^{2+} between 0.8 and 5 mM. When expressed in terms of the relative proliferation compared to the condition without Mg^{2+} (Fig. 2B), the relative stimulations by the Mg^{2+} were higher at 0.1 mM Ca^{2+} compared to 0.5 mM Ca^{2+} and 1 mM Ca^{2+} ($P<0.001$, Anova), and a significant difference between 0.5 and 1 mM Ca^{2+} ($P<0.05$, Anova) was still observed (Table 1). It should be noted that cell proliferation did not reach control levels (1 mM Ca^{2+} and 0.8 mM Mg^{2+}) with higher concentrations of Mg^{2+} (5 mM) under low Ca^{2+} levels. As shown in Fig. 3A, concentrations of Ca^{2+} below 0.5 mM reduced cell proliferation at all concentrations of Mg^{2+}. No difference of cell proliferation was observed for concentration of 0.5 mM Ca^{2+} and above. When expressed in terms of the relative proliferation compared to the condition with low Ca^{2+} (0.1 mM) (Fig. 3B), the relative stimulations by the Ca^{2+} were higher for the condition without Mg^{2+} and 0.025 mM Mg^{2+} ($P<0.0001$, Anova), 0.1 mM Mg^{2+} ($P<0.0003$, Anova), 0.4 mM Mg^{2+} ($P<0.05$, Anova) compared to 0.8 mM (Table 1). It should be noted that cell proliferation did not reach control levels (1 mM Ca^{2+} and 0.8 mM Mg^{2+}) with higher concentrations of Ca^{2+} (2 mM) under conditions without extracellular Mg^{2+}. Protein quantification also confirmed the relative reduction of cell proliferation by low extracellular Ca^{2+} and Mg^{2+} (data not shown). As shown in Table 2, osteoblastic phenotype marker such as alkaline phosphatase activity was not different between conditions that reduced cell proliferation. Similar reduction of cell proliferation under

extracellular low Mg^{2+} and Ca^{2+} conditions was obtained with two other human osteoblast-like cells, namely SaOs and U2 OS (data not shown). The effects of reducing extracellular Ca^{2+} and Mg^{2+} on the proliferation were reversible since exposing Ca^{2+} and Mg^{2+}-depleted cells, which showed growth arrest after 48 h of incubation (as shown in the Fig. 2 and 3), to DMEM/F12 (1 mM Ca^{2+} and 0.8 mM Mg^{2+}) for a further culture period of 96 h with FBS restored the cell proliferation (relative MTT activity of 1.97 ± 0.29 vs 1.06 ± 0.12).

To confirm that the reduction of Ca^{2+} and Mg^{2+} of the culture media lead to a decrease of osteoblastic cell proliferation, we measured DNA synthesis of MG-63 cells under representative culture conditions which allowed reduction of cell proliferation evaluated by MTT assays. Figure 4 showed that DNA synthesis was significantly decreased at 24 and 48 h under culture conditions of low Mg^{2+} for both 0.1 and 1 mM Ca^{2+} whereas significant stimulation of DNA synthesis was obtained with PDGF used as positive control for cell proliferation. DNA synthesis was not different from control condition DMEM/F12 for concentration of Mg^{2+} of 0.8 mM with 0.1 mM Ca^{2+} and of 0.4 mM Mg^{2+} with 1 mM Ca^{2+}. Therefore, our DNA synthesis data agree with the MTT assays. Furthermore, the effect of low Mg^{2+} and Ca^{2+} on cell proliferation was confirmed by cell counts (Table 3).

Expression of TRPM and channel activation under low extracellular Mg^{2+} and Ca^{2+} levels.

In order to investigate the potential implication of TRPM6 and TRPM7 in compensatory mechanisms to maintain cellular ion homeostasis, we quantified their expression levels from cells maintained for 48 h under low concentrations of Mg^{2+} and/or Ca^{2+}. As shown in Fig. 5, the expression of TRPM7 increased by approximately 2-fold in the absence of Mg^{2+} and low concentration of Ca^{2+} whereas no difference was noticed for the expression of TRPM6.

In order to determine the influence of low extracellular levels of Ca^{2+} and Mg^{2+} on the activation of channels for the maintenance of cellular ion homeostasis, we performed intracellular Ca^{2+} and Mg^{2+} measurements with Fluo3 and Magnesium Green. As shown in Fig. 6A (left panel: inset), the addition of calcium to cells with prior incubation of 2 h in Ca^{2+}-free and 0.8 mM Mg^{2+} incubation medium has not effect on the intracellular Ca^{2+} level of MG-63 cells. Similar results were obtained for cells with prior incubation in normal 1mM Ca^{2+} and 0.8 mM Mg^{2+} incubation medium (data not shown). On the other hand, prior incubation of cells in 1 mM Ca^{2+} and Mg^{2+}-free media for 2 h resulted in the observation of a Ca^{2+} influx upon addition of the ion to the incubation medium (Fig. 6A, left panel) suggesting that channels were activated by previous incubation conditions without Mg^{2+}. Accordingly, the addition of extracellular Mg^{2+} resulted in an increase of intracellular Mg^{2+} in cells pre-incubated for 2 h in 1 mM Ca^{2+} and Mg^{2+}-free medium (Fig. 6A, right panel). Furthermore, no significant augmentation of intracellular Mg^{2+} was observed upon

addition of extracellular Mg^{2+} with cells pre-incubated in Ca^{2+}-free and 0.8 mM Mg^{2+} incubation medium (Fig. 6A, right panel: inset) or in normal 1 mM Ca^{2+} and 0.8 mM Mg^{2+} medium (data not shown). We took advantage of the interference strategy by siRNA to evaluate the importance of TRPM7 in these Ca^{2+} and Mg^{2+} influx. The Fig. 6B showed that two specific siRNA for TRPM7 (si-TRPM7(1) and si-TRPM7(2)) reduced the expression by approximately 60% after 48 h of incubation whereas no modification of the expression of TRPM6 was noticed. The reduction of TRPM7 expression prevented the Ca^{2+} influx induced by prior incubation of MG-63 cells in Mg^{2+}-free conditions (Fig. 6C, left panel). On the other hand, store-operated Ca^{2+} channel antagonist SKF-96365 was without effect the Ca^{2+} influx indicating that TRPC channels are not involved (Fig. 6C, right panel). Similarly, Mg^{2+} influx was prevented when cells were transfected with siRNA against TRPM7 (data not shown).

Cell proliferation under condition of reduced TRPM7 expression

In order to determine the importance of TRPM7 channels in the proliferation of osteoblastic cells, MTT assays were performed with cells transfected with specific siRNA against TRPM7. The Fig. 7A and B showed that the proliferation of osteoblastic-like MG-63 and U2 OS cells respectively was inhibited by 60-75% with specific siRNAs for TRPM7.

Discussion

Influx of both Ca^{2+} and Mg^{2+} is solicited for numerous processes implicated in cell proliferation (Berridge *et al.* 2000; Rubin 2005). In contrast to agonist-stimulated ion influx that is generally rapid and transient, spontaneously activated ionic channel regulated by intracellular ion availability are more likely involved in the progression of cell cycle. Accordingly, TRPM6 and TRPM7 channel activity has been shown to be regulated by intracellular Mg (Fleig and Penner 2004). Therefore, we investigated the involvement of such channels in the proliferation of osteoblastic cells.

We show for the first time the expression of TRPM6 and TRPM7 channels in human osteoblast-like cells. The expression was revealed for cells of recognized preosteoblast phenotype (MG-63 and U2OS) and for more mature osteoblastic cells (SaOS). Expression of TRPM7 has been reported in neurons (Aarts *et al.* 2003), lymphocytes (Perraud *et al.* 2004), cardiac myocytes (Gwanyanya *et al.* 2004), vascular smooth muscle cells (Beech 2005), and is thought to be widely expressed. TRPM7 channels have been associated with cell viability (Nadler *et al.* 2001), cellular Mg homeostasis (Schmitz *et al.* 2003), anoxic neuronal cell death (Aarts *et al.* 2003), intestinal pacemaking activity (Kim *et al.* 2005), cell proliferation of retinoblastoma cells (Hanano *et al.* 2004), vascular smooth muscle cell proliferation and response to fluid flow (Beech 2005).

As Ca^{2+} and Mg^{2+} conducting channels, TRPM6 and TRPM7 may be involved in the intracellular homeostasis of both ions that have been associated with important cell functions. We observed significant reduction of osteoblastic cell proliferation under culture conditions of low extracellular Mg^{2+} or Ca^{2+} concentrations. The decrease of cell proliferation was amplified by the concomitant reduction of extracellular Mg and Ca in the culture media. Accumulating evidence, from bacteria to human cells, points to a universal role for Mg^{2+} in controlling numerous processes associated with the cell cycle (for a review, (Rubin 2005)). It has been proposed that Ca^{2+} and Mg^{2+} act in concert via a common mechanism. Although our results agree with the important role of Mg^{2+} in cell proliferation, both ions seem essential for optimal cell proliferation since higher Mg^{2+} concentration (5 mM) or higher Ca^{2+} concentration (2 mM) did not compensate each other. Therefore, part of the progression of the cell proliferation is ensured by both ions acting probably together via a common mechanism but distinct mechanisms are revealed for each ion to reach optimal cell proliferation. In that regards, TRPM6 and TRPM7 channels may promote influx of both Ca^{2+} and Mg^{2+} to assure the accomplishment of respective processes of both ions for cell proliferation. Accordingly, the reduction of TRPM7 expression by 60% in osteoblastic cells using the interference by siRNA led to a drastic reduction (around 60-75%) of cell proliferation. Similar correlation of TRPM7 and cell proliferation was reported by Hanano et al in retinoblastoma cells (Hanano *et al.* 2004), and by He et al in vascular smooth muscle cells (He *et al.* 2005). Therefore, TRPM6 could not compensate for the reduction of TRPM7 expression for osteoblastic cell proliferation. Schmitz et al

(2005) have reported heteromeric formation of TRPM6 channels with TRPM7 and that TRPM6 requires TRPM7 for cell surface expression. In addition, it was shown that TRPM7 deficiency cannot be complemented by TRPM6. Moreover, Chubanov et al (2004) reported similar requirement of TRPM6/TRPM7 association.

In view of the increase of TRPM7 expression that we revealed under low Mg^{2+} and Ca^{2+} levels, TRPM7 may be part of a compensatory mechanism in view of the gradual intracellular deficit that arise from low concentrations of extracellular Mg^{2+} and Ca^{2+} which agree with the reported ionic conductance of TRPM7 for Ca^{2+} and Mg^{2+}. Surprisingly, osteoblastic TRPM6 expression was not modified by reducing extracellular concentrations of both ions. Upregulation of renal TRPM6 mRNA levels by low Mg diet has been reported whereas similar upregulation of intestinal TRPM6 expression was observed by enriched Mg diet (Groenestege *et al.* 2006). On the other hand, TRPM7 expression was not modified under both conditions. However, it could not be excluded that renal and intestinal expression of TRPM6 may be regulated in vivo by indirect mechanism and not by Mg^{2+} directly. Such indirect mechanisms may implicate endocrine regulation since the expression of TRPM6 has been shown to be increased by 17β-estradiol (Groenestege *et al.* 2006). Recently, Goytain & Quamme have described the cloning and the characterization of numerous plasma membrane Mg^{2+} transporters designated SLC41A1 (Goytain and Quamme 2005b), SLC41A2 (Goytain and Quamme 2005c), MagT1 (Goytain and Quamme 2005d) and ACDP2 (Goytain and Quamme 2005a). The renal expression of SLC41A1, MagT1 and

ACDP2 was shown to be upregulated in mice under hypomagnesic conditions. Also, the expression of MagT1 and ACDP2 was upregulated in distal tubule kidney cells (MDCT) by reduced extracellular Mg^{2+} level. Our current results indicate that TRPM7 could be added to this list of Mg^{2+} transporters that are upregulated by reducing extracellular Mg^{2+} levels. No information is available until now about the expression of these Mg^{2+} transporters in osteoblastic cells. However, given that reducing TRPM7 expression in osteoblastic cells results in the reduction of cell proliferation, it is proposed that these Mg^{2+} transporters are not expressed in osteoblastic cells or even in their presence, they can not compensate for the reduction of TRPM7 expression. Accordingly, Sahni et al (2007) have reported that although TRPM7-deficient DT40 cells express the SLC41A2 transporter, its endogenous expression level do not compensate for the reduction of cell proliferation.

Although conditions of restricted Mg^{2+} levels have been shown to modify eukaryotic gene expression, the mechanism is unknown. In prokaryote, a two-component system, namely PhoP-PhoQ, has been documented to mediate the adaptation to Mg-limiting environments, and regulates numerous cellular activities in several gram-negative species (for a review (Groisman 2001)). PhoP-PhoQ constitutes the first example of a regulatory system that uses extracellular Mg^{2+} as a primary signal. Growth in micromolar concentrations of Mg^{2+} promotes transcription of PhoP-activated genes in a PhoP-and PhoQ-dependent manner. In addition to Mg^{2+}, Ca^{2+} and manganese can repress transcription of PhoP-activated genes. Consistent with Mg^{2+} and Ca^{2+} being

the physiological signals controlling the PhoP-PhoQ system, several PhoP-dependent phenotypes are regulated by these divalent cations in wild-type microorganisms. Especially the PhoP-PhoQ system regulates the transcription of mgtA, mgtB and mgtC genes, which encode P-type ATPases that transport Mg^{2+}. The existence of homologous systems in eukaryotic cells has to be investigated. Maier et al. (2004) have identified by cDNA array several transcripts modulated by exposure to low Mg^{2+}, such as c-src, ezrin, CD9, cytohesin and zyxin. Such cellular response to Mg^{2+} reduction was suggested to alter the endothelial adhesion to substrates and migration, and thereby promote atherosclerosis, thrombosis and hypertension associated to Mg^{2+} deficiency. Therefore, conditions of restricted Mg^{2+} levels potentially modify eukaryotic cell phenotype. In that regards Ca^{2+} and Mg^{2+} may act as independent repressors for the transcription of TRPM7 channels in osteoblastic cells as suggested by our results (Fig. 5). Given that cell proliferation was restored, although without being optimal, by Ca^{2+} and Mg^{2+} when the extracellular concentration of the counterpart ion was reduced, the independent repression of TRPM7 expression by Ca^{2+} and Mg^{2+} may be the result of the Ca^{2+}- and Mg^{2+}-transporting capacity of TRPM7 with the fact that both ions act in part via common mechanisms on the cell proliferation.

Electrophysiological studies have indicated that TRPM7-mediated currents (Monteilh-Zoller *et al.* 2003), also known as MIC (Mg^{2+}-inhibited cation) current or MagNum (Mg^{2+}-nucleotide-inhibited metal) current (Nadler *et al.* 2001; Prakriya and

Lewis 2003), are regulated by the cytosolic Mg^{2+}. When osteoblastic cells were incubated in Mg^{2+}-deficient medium for 2 h, Ca^{2+} and Mg^{2+} influx were observed upon its addition to the incubation medium indicating that channels were activated under latter condition. Similar Ca^{2+} and Mg^{2+} influx were not seen for cells transfected with specific siRNA against TRPM7 suggesting that TRPM7 in osteoblastic cells were activated by low concentrations of extracellular Mg^{2+}. Although the transfection with siRNA against TRPM7 led to a 60% reduction of TRPM7 expression, the Ca^{2+} and Mg^{2+} influx induced by prior incubation of cells in Mg^{2+}-deficient medium were mostly prevented. Since TRP channels are usually formed by tetramerization, a threshold of TRPM7 expression may be essential to obtain functional channels at the plasma membrane.

The activation of TRPM7 was not instantaneous following the reduction of extracellular Mg^{2+} since no Ca^{2+} influx was seen upon addition of extracellular Ca^{2+} to cells previously transferred in Ca^{2+}-free and Mg^{2+}-free HBSS for few minutes. In addition, the absence of Ca^{2+} influx from cells incubated in HBSS without Ca^{2+} with 0.8 mM Mg^{2+} further support that Mg^{2+} is the main ion which regulated TRPM7 activity. Latter results agree with the activation of TRPM7 following the gradual reduction of intracellular Mg^{2+}.

Magnesium deficiency is not uncommon among the general population: its intake has decreased over the years in a significant proportion of the population, especially in the western world. Many important human pathologies such as hypertension, heart

failure, several nervous system, muscle diseases and atherosclerosis have been associated with a decrease of Mg^{2+} availability (Laires *et al.* 2004). In addition, co-existing disorders that impair intestinal Mg^{2+} absorption and/or are associated with renal Mg^{2+} loss, such as malabsorption syndromes, alcoholism, diabetes mellitus, and drugs (e.g., diuretics), would place an individual at even greater risk for Mg^{2+} depletion. It should be noted however that serum Mg^{2+} levels do not reflect actual body stores of Mg^{2+} since blood levels are kept within the normal range at the expense of other tissues, especially bones. In this regard epidemiological studies provide a link associating insufficient dietary Mg^{2+} intake in humans with low bone mass and osteoporosis (for a review (Rude and Gruber 2004)). Experimental Mg^{2+} deficiency in animal models resulted in impaired bone growth, osteopenia, and increased skeletal fragility. Magnesium depletion causes a decrease in both osteoblast number and osteoclast activity with the development of a form of 'aplastic bone disease'. Moreover, genetic hypomagnesemia with renal Mg^{2+} wasting lead to low bone mass (Kantorovich *et al.* 2002). Our current results indicate that extracellular Mg^{2+} deficit *in vitro* reduce osteoblast proliferation, an effect amplified by low extracellular Ca^{2+} concentrations. Such reduction in osteoblast cell proliferation would lead to inappropriate bone formation and inadequate regulation of resorption resulting in the development of osteoporosis.

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Table 1. Relative stimulation of cell proliferation as a function of Ca^{2+} and Mg^{2+} levels in the culture media

Conditions	**0.8 mM Mg^{2+} vs 0 mM Mg^{2+}**	**Conditions**	**1 mM Ca^{2+} vs 0.1 mM Ca^{2+}**
0.1 mM Ca^{2+}	1.79 ± 0.17^{a}	0 mM Mg^{2+}	1.66 ± 0.16^{c}
0.5 mM Ca^{2+}	1.47 ± 0.05^{b}	0.025 mM Mg^{2+}	1.55 ± 0.14^{c}
1 mM Ca^{2+}	1.35 ± 0.10	0.1 mM Mg^{2+}	1.50 ± 0.04^{d}
		0.4 mM Mg^{2+}	1.33 ± 0.10^{e}
		0.8 mM Mg^{2+}	1.24 ± 0.09

$^{a}P<0.0001$, $^{b}P<0.05$ compared to condition with 1 mM Ca^{2+} (Student T test)

$^{c}P<0.0001$, $^{d}P<0.0003$, $^{e}P<0.05$ compared to condition with 0.8 mM Mg^{2+} (Student T test)

Table 2. Effect of extracellular Mg^{2+} and Ca^{2+} on alkaline phosphatase activity

	Alkaline phosphate* (nmol PNP/1h/mg protein)				
Condition (Calcium)	**0.1 mM**	**0.25 mM**	**0.5 mM**	**1 mM**	**2 mM**
0 mM Mg^{2+}	591.6±48.4	559.4±85.9	539.2±78.9	678.6±115.6	579.1±66.8
Condition (Magnesium)**	**0 mM**	**0.1 mM**	**0.4 mM**	**0.8 mM**	**5 mM**
0.1 mM Ca^{2+}	591.6±48.4	658.8±56.4	624.8±53.4	583.4±47.4	615.4±126.2

* Mean ± SD of alkaline phosphatase activity

**Alkaline phosphatase activity from condition with 0.8 mM Mg^{2+} and 1 mM Ca^{2+} (519.9±90.4 nmol PNP/1h/mg protein) not different from DMEM/F12 (496.2±61.6 nmol PNP/1h/mg protein)

Table 3. Effect of low concentrations of extracellular Mg^{2+} and Ca^{2+} on cell counts

	Cell counts				
Condition* (Magnesium)	**0 mM**	**0.025 mM**	**0.1 mM**	**0.8 mM**	**2 mM**
0.1 mM Ca^{2+}	33.6±4.6*	52.5±7.6^{c}(1.6)	57.5±18.3^{d}(1.7)	71.8±8.8^{b}(2.1)	73.5±17.7^{a}(2.2)
Condition (Calcium)	**0.1 mM**	**0.25 mM**	**1 mM**		
0 mM Mg^{2+}	33.6±4.6	54.0±5.3^{e}[1.6]	60.4±6.9^{e}[1.8]		

*cells±SD X1000 after 48 h of incubation

$^{a}P<0.01$, $^{b}P<0.001$, $^{c}P<0.02$, $^{d}P<0.05$ when compared to condition without Mg^{2+}

$^{e}P<0.001$ when compared to condition with 0.1 mM Ca^{2+}

() ratio vs 0 mM Mg^{2+}

[] ratio vs 0.1 mM Ca^{2+}

Reference

Aarts M, Iihara K, Wei WL, Xiong ZG, Arundine M, Cerwinski W, MacDonald JF, Tymianski M (2003) A key role for TRPM7 channels in anoxic neuronal death. Cell **115**, 863-877.

Beech DJ (2005) Emerging functions of 10 types of TRP cationic channel in vascular smooth muscle. Clin. Exp. Pharmacol. Physiol. **32**, 597-603.

Bergman RJ, Gazit D, Kahn AJ, Gruber H, McDougall S, Hahn TJ (1996) Age-related changes in osteogenic stem cells in mice. J. Bone Miner. Res. **11**, 568-577.

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. **1**, 11-21.

Brockstedt H, Kassem M, Eriksen EF, Mosekilde L, Melsen F (1993) Age- and sex-related changes in iliac cortical bone mass and remodeling. Bone **14**, 681-691.

Chan GK, Duque G (2002) Age-related bone loss: old bone, new facts. Gerontology **48**, 62-71.

Chen TL (2004) Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. Bone **35**, 83-95.

Chubanov V, Waldegger S, Schnitzler M, Vitzthum H, Sassen MC, Seyberth HW, Konrad M, Gudermann T (2004) Disruption of TRPM6/TRPM7 complex formation by a mutation in the TRPM6 gene causes hypomagnesemia with secondary hypocalcemia. Proc. Natl. Acad. Sci. USA **101**, 2894-2899.

Clapham DE, Runnels LW, Strubing C (2001) The TRP ion channel family. Nat. Rev. Neurosci. **2**, 387-396.

D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA (1999) Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. J. Bone Miner. Res. **14**, 1115-1122.

Duncan RL, Akanbi KA, Farach-Carson MC (1998) Calcium signals and calcium channels in osteoblastic cells. Semin. Nephrol. **18**, 178-190.

Fleig A, Penner R (2004) The TRPM ion channel subfamily: molecular, biophysical and functional features. Trends Pharmacol. Sci. **25**, 633-639.

Goytain A, Quamme GA (2005a) Functional characterization of ACDP2 (ancient conserved domain protein), a divalent metal transporter. Physiol. Genomics **22**, 382-389.

Goytain A, Quamme GA (2005b) Functional characterization of human SLC41A1, a Mg^{2+} transporter with similarity to prokaryotic MgtE Mg^{2+} transporters. Physiol. Genomics **21**, 337-342.

Goytain A, Quamme GA (2005c) Functional characterization of the human solute carrier, SLC41A2. Biochem. Biophys. Res. Commun. **330**, 701-705.

Goytain A, Quamme GA (2005d) Identification and characterization of a novel mammalian Mg^{2+} transporter with channel-like properties. BMC. Genomics **6**, 48.

Groenestege WM, Hoenderop JG, van den HL, Knoers N, Bindels RJ (2006) The epithelial Mg^{2+} channel transient receptor potential melastatin 6 is regulated by dietary Mg^{2+} content and estrogens. J. Am. Soc. Nephrol. **17**, 1035-1043.

Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183**, 1835-1842.

Gwanyanya A, Amuzescu B, Zakharov SI, Macianskiene R, Sipido KR, Bolotina VM, Vereecke J, Mubagwa K (2004) Magnesium-inhibited, TRPM6/7-like channel in cardiac myocytes: permeation of divalent cations and pH-mediated regulation. J. Physiol. **559**, 761-776.

Hanano T, Hara Y, Shi J, Morita H, Umebayashi C, Mori E, Sumimoto H, Ito Y, Mori Y, Inoue R (2004) Involvement of TRPM7 in cell growth as a spontaneously activated Ca^{2+} entry pathway in human retinoblastoma cells. J. Pharmacol. Sci. **95**, 403-419.

Harteneck C (2005) Function and pharmacology of TRPM cation channels. Naunyn Schmiedebergs Arch. Pharmacol. **371**, 307-314.

Harteneck C, Plant TD, Schultz G (2000) From worm to man: three subfamilies of TRP channels. Trends Neurosci. **23**, 159-166.

He Y, Yao G, Savoia C, Touyz RM (2005) Transient receptor potential melastatin 7 ion channels regulate magnesium homeostasis in vascular smooth muscle cells: role of angiotensin II. Circ. Res. **96**, 207-215.

Iqbal J, Zaidi M (2005) Molecular regulation of mechanotransduction. Biochem. Biophys. Res. Commun. **328**, 751-755.

Kantorovich V, Adams JS, Gaines JE, Guo X, Pandian MR, Cohn DH, Rude RK (2002) Genetic heterogeneity in familial renal magnesium wasting. J. Clin. Endocrinol. Metab. **87**, 612-617.

Kassem M, Ankersen L, Eriksen EF, Clark BF, Rattan SI (1997) Demonstration of cellular aging and senescence in serially passaged long-term cultures of human trabecular osteoblasts. Osteoporos. Int. **7**, 514-524.

Kato H, Matsuo R, Komiyama O, Tanaka T, Inazu M, Kitagawa H, Yoneda T (1995) Decreased mitogenic and osteogenic responsiveness of calvarial osteoblasts isolated from aged rats to basic fibroblast growth factor. Gerontology **41 Suppl 1**, 20-27.

Kim BJ, Lim HH, Yang DK, Jun JY, Chang IY, Park CS, So I, Stanfield PR, Kim KW (2005) Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. Gastroenterology **129**, 1504-1517.

Kotev-Emeth S, Savion N, Pri-chen S, Pitaru S (2000) Effect of maturation on the osteogenic response of cultured stromal bone marrow cells to basic fibroblast growth factor. Bone **27**, 777-783.

Kragstrup J, Melsen F, Mosekilde L (1983) Thickness of bone formed at remodeling sites in normal human iliac trabecular bone: variations with age and sex. Metab. Bone Dis. Relat. Res. **5**, 17-21.

Laires MJ, Monteiro CP, Bicho M (2004) Role of cellular magnesium in health and human disease. Front Biosci. **9**, 262-276.

Liang CT, Barnes J, Seedor JG, Quartuccio HA, Bolander M, Jeffrey JJ, Rodan GA (1992) Impaired bone activity in aged rats: alterations at the cellular and molecular levels. Bone **13**, 435-441.

Mackie EJ (2003) Osteoblasts: novel roles in orchestration of skeletal architecture. Int. J. Biochem. Cell Biol. **35**, 1301-1305.

Maier JA, Malpuech-Brugere C, Zimowska W, Rayssiguier Y, Mazur A (2004) Low magnesium promotes endothelial cell dysfunction: implications for atherosclerosis, inflammation and thrombosis. Biochim. Biophys. Acta **1689**, 13-21.

Martinez ME, del Campo MT, Medina S, Sanchez M, Sanchez-Cabezudo MJ, Esbrit P, Martinez P, Moreno I, Rodrigo A, Garces MV, Munuera L (1999) Influence of skeletal site of origin and donor age on osteoblastic cell growth and differentiation. Calcif. Tissue Int. **64**, 280-286.

Minke B, Cook B (2002) TRP channel proteins and signal transduction. Physiol. Rev. **82**, 429-472.

Monteilh-Zoller MK, Hermosura MC, Nadler MJ, Scharenberg AM, Penner R, Fleig A (2003) TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. J. Gen. Physiol. **121**, 49-60.

Montell C, Birnbaumer L, Flockerzi V (2002) The TRP channels, a remarkably functional family. Cell **108**, 595-598.

Moreau R, Aubin R, Lapointe JY, Lajeunesse D (1997) Pharmacological and biochemical evidence for the regulation of osteocalcin secretion by potassium channels in human osteoblast-like MG-63 cells. J. Bone Miner. Res. **12**, 1984-1992.

Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurosaki T, Kinet JP, Penner R, Scharenberg AM, Fleig A (2001) LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. Nature **411**, 590-595.

Neidlinger-Wilke C, Stalla I, Claes L, Brand R, Hoellen I, Rubenacker S, Arand M, Kinzl L (1995) Human osteoblasts from younger normal and osteoporotic donors show differences in proliferation and TGF beta-release in response to cyclic strain. J. Biomech. **28**, 1411-1418.

Parfitt AM (1991) Bone Forming Cells in Clinical Conditions. In 'Bone, the osteoblast and osteocyte'. (Ed. Hall BK) pp. 351-426. (London: Telford Press)

Perraud AL, Knowles HM, Schmitz C (2004) Novel aspects of signaling and ion-homeostasis regulation in immunocytes. The TRPM ion channels and their potential role in modulating the immune response. Mol. Immunol. **41**, 657-673.

Peterson WJ, Tachiki KH, Yamaguchi DT (2004) Serial passage of MC3T3-E1 cells down-regulates proliferation during osteogenesis in vitro. Cell Prolif. **37**, 325-336.

Pfeilschifter J, Diel I, Pilz U, Brunotte K, Naumann A, Ziegler R (1993) Mitogenic responsiveness of human bone cells in vitro to hormones and growth factors decreases with age. J. Bone Miner. Res. **8**, 707-717.

Prakriya M, Lewis RS (2003) CRAC channels: activation, permeation, and the search for a molecular identity. Cell Calcium **33**, 311-321.

Quarto R, Thomas D, Liang CT (1995) Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calcif. Tissue Int. **56**, 123-129.

Roholl PJ, Blauw E, Zurcher C, Dormans JA, Theuns HM (1994) Evidence for a diminished maturation of preosteoblasts into osteoblasts during aging in rats: an ultrastructural analysis. J. Bone Miner. Res. **9**, 355-366.

Romani AM, Scarpa A (2000) Regulation of cellular magnesium. Front Biosci. **5**, D720-D734.

Rubin H (2005) The membrane, magnesium, mitosis (MMM) model of cell proliferation control. Magnes. Res. **18**, 268-274.

Rude RK, Gruber HE (2004) Magnesium deficiency and osteoporosis: animal and human observations. J. Nutr. Biochem. **15**, 710-716.

Sahni J, Nelson B, Scharenberg AM (2007) SLC41A2 encodes a plasma-membrane Mg^{2+} transporter. Biochem. J. **401**, 505-513.

Schmitz C, Dorovkov MV, Zhao X, Davenport BJ, Ryazanov AG, Perraud AL (2005) The channel kinases TRPM6 and TRPM7 are functionally nonredundant. J. Biol. Chem. **280**, 37763-37771.

Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, Kurosaki T, Fleig A, Scharenberg AM (2003) Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. Cell **114**, 191-200.

Stenderup K, Justesen J, Clausen C, Kassem M (2003) Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone **33**, 919-926.

Wolf FI, Fasanella S, Tedesco B, Torsello A, Sgambato A, Faraglia B, Palozza P, Boninsegna A, Cittadini A (2004) Regulation of magnesium content during proliferation of mammary epithelial cells (HC-11). Front Biosci. **9**, 2056-2062.

Legend of the figures

Fig. 1. Analysis of genetic expression of TRPM6 and TRPM7 channels in human osteoblast-like cells. Total RNA from MG-63, SaOS and U2 OS cells was isolated and subjected to RT-PCR using specific primers for TRPM6 (NM_017662) and TRPM7 (NM_017672) channels. Results are representative data obtained from 3 independent isolations of RNA. Ladder of 100 bp.

Fig. 2. Effect of extracellular Mg^{2+} on MG-63 proliferation. Cells were incubated for 48 h in culture media containing different concentrations of Ca^{2+} and increasing concentrations of Mg^{2+}, and cell proliferation was determined by MTT assays. Results are expressed as (A) the ratio of the absorbance compared to the initial MTT or (B) as the relative effect of Mg^{2+} compared to the condition without Mg^{2+}. Values for normal DMEM/F12 were 2.12 ± 0.21 relative to the initial MTT. Data are means ± SEM of at least 3 individual experiments.

Fig. 3. Effect of extracellular Ca^{2+} on MG-63 proliferation. Cells were incubated for 48 h in culture media containing different concentrations of Mg^{2+} and increasing concentrations of Ca^{2+}, and cell proliferation was determined by MTT assays. Results are expressed as (A) the ratio of the absorbance compared to the initial MTT or (B) as the relative effect of Ca^{2+} compared to the condition with 0.1 mM Ca^{2+}. Values for normal DMEM/F12 were 2.12 ± 0.21. Data are means ± SEM of at least 3 individual experiments.

Fig. 4. Effect of extracellular Mg^{2+} and Ca^{2+} on DNA synthesis. Cells were incubated for 24 or 48 h in DME/F12 containing different concentrations of Mg^{2+} and Ca^{2+}. The evaluation of DNA synthesis was performed by BrdU incorporation as described in Material and Methods. As positive control for the stimulation

of DNA synthesis, cells were incubated with 25 ng/ml PDGF-BB in DMEM/F12. Values are the mean ± SEM of the percentage compared to DMEM/F12 medium from 3-4 individual experiments. $^{a}P<0.05$, $^{b}P<0.02$, $^{c}P<0.01$, $^{d}P<0.001$ compared to DMEM/F12.

Fig. 5. Expression of TRPM6 and TRPM7 in osteoblast-like cells under Mg^{2+}- and/or Ca^{2+}-reduced culture condition. MG-63 cells were incubated for 48 h in DME/F12 containing 1 mM Ca^{2+} without Mg^{2+}, in DME/F12 containing 0.8 mM Mg^{2+} and 0.1 mM Ca^{2+}, in DME/F12 containing 0.1 mM Ca^{2+} without Mg^{2+} or in DMEM/F12 (1 mM Ca^{2+} and 0.8 mM Mg^{2+}). Total RNA was isolated from cells cultured under previous conditions and the levels of TRPM6 and TRPM7 transcripts were determined by semi-quantitative RT-PCR (A) or real-time PCR (B) as described in Material and Methods. The Relative levels of TRPM7 expression compared to control condition (DMEM/F12) are expressed as the mean ± SEM of 3 independent experiments. **P<0.008, Student T test

Fig. 6. Activation of TRPM7 under low extracellular Mg^{2+} levels. A) Cells were loaded for 2 h incubation period with Fluo-3 or Magnesium Green in HBSS with 1mM Ca^{2+} without Mg^{2+} or HBSS without Ca^{2+} with 0.8 mM Mg^{2+} (right panel: inset). Thereafter cells were transferred to Ca^{2+}-free and Mg^{2+}-free HBSS and Ca^{2+} (left panel) or Mg^{2+} (right panel) were added to the incubation medium (final concentration of 3 mM). Measurements of intracellular Ca^{2+} or Mg^{2+} were performed as described in the Material and Methods section. B) MG-63 cells were transfected with specific siRNA against TRPM7 for 48 h. RT-PCR and RQ-PCR were performed to evaluate the expression levels of TRPM6, TRPM7 and beta-2-microglobulin. **P<0.001. C) Cells were transfected with si-TRPM7 (2) for 48 h and loaded for 2 h incubation period with Fluo-3 in HBSS with 1mM Ca^{2+} without Mg^{2+}. Thereafter cells were transferred to Ca^{2+}-free and Mg^{2+}-free HBSS and Ca^{2+}

were added to the incubation medium (final concentration of 3 mM). (right panel: non-transfected cells loaded with Fluo-3 in HBSS with 1 mM Ca^{2+} without Mg^{2+} were first incubated with 30 μM SKF-96365 and Ca^{2+} was added to the incubation medium). Data are means ± SEM of 3 to 4 individual experiments with cumulating analysis of between 10 to 20 cells per field.

Fig. 7. Effect of reducing TRPM7 expression on osteoblast-like cell proliferation. Cells were transfected with specific siRNAs against TRPM7 (siTRPM7(1) and si-TRPM7(2)) or control negative siRNA (siRNA(-)) and cell proliferation was determined after 72 h for MG-63 cells (A) or U2 OS cells (B) and compared to the MTT activity following 24h of incubation with siRNA (MTT initial). Data are means ± SEM of 3 individual experiments. *$P<0.05$ and **$P<0.001$ compared to cell proliferation with siRNA(-).

Fig. 1. Analysis of genetic expression of TRPM6 and TRPM7 channels in human osteoblast-like cells.

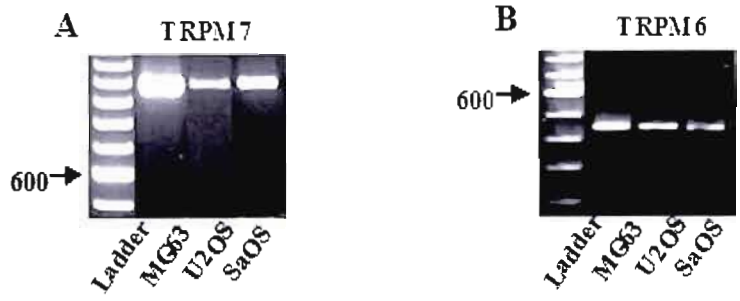


Fig. 2. Effect of extracellular Mg^{2+} on MG-63 proliferation

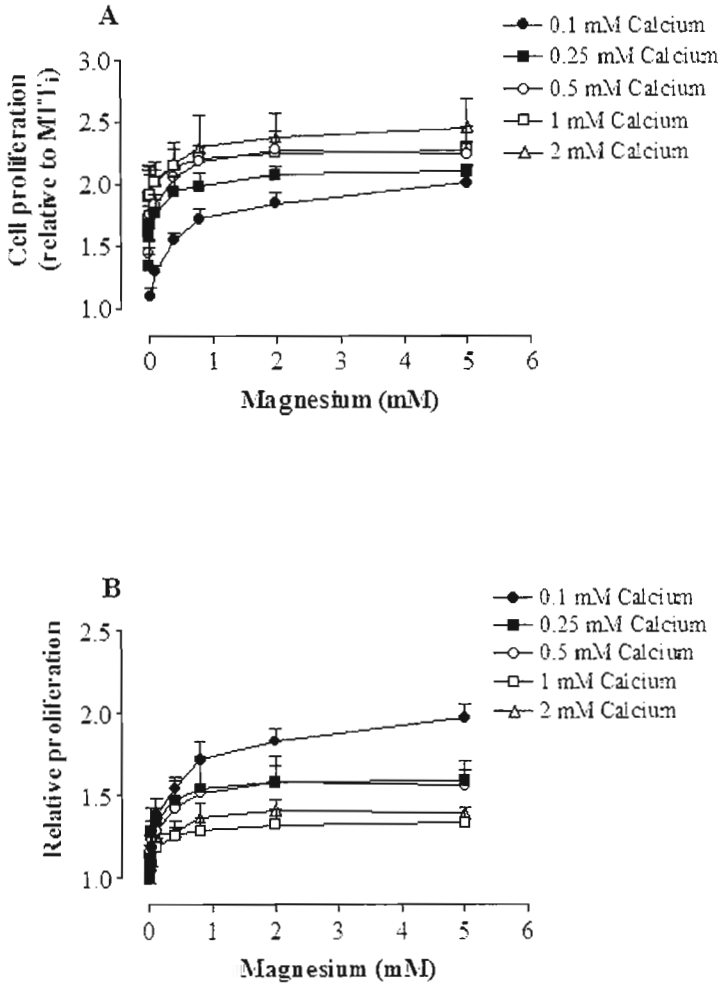


Fig. 3. Effect of extracellular Ca^{2+} on MG-63 proliferation.

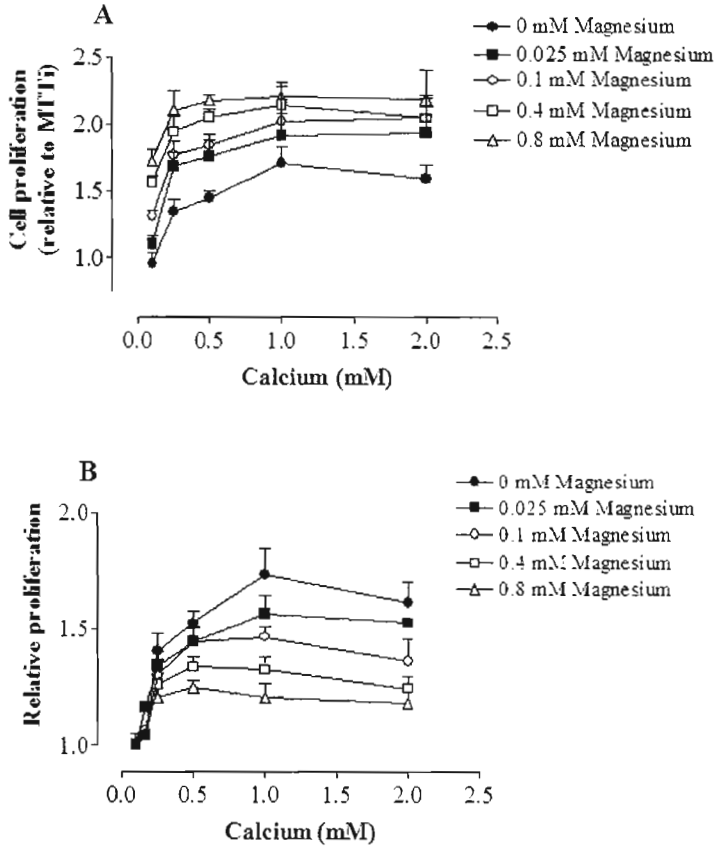


Fig. 4. Effect of extracellular Mg^{2+} and Ca^{2+} on DNA synthesis.

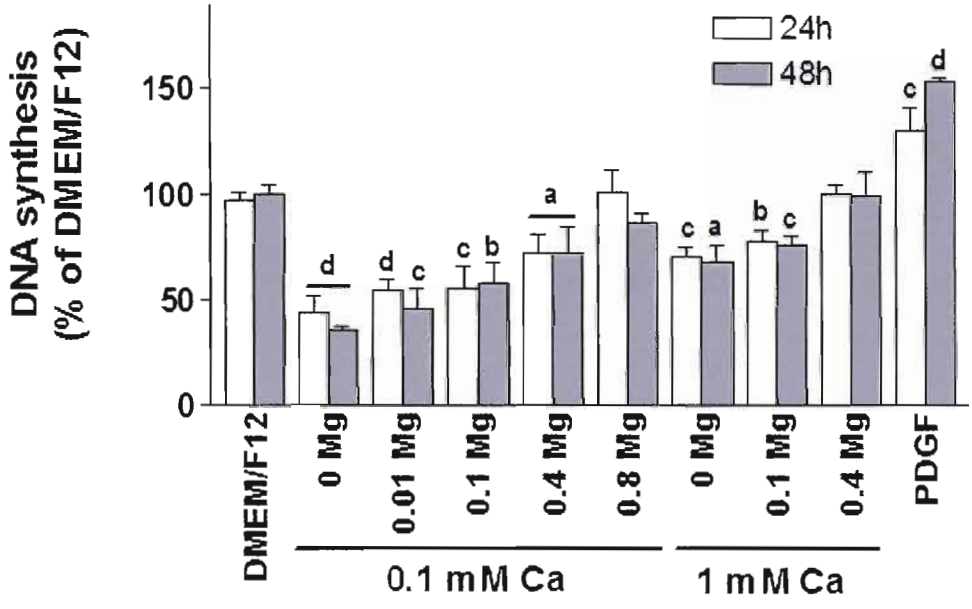


Fig. 5. Expression of TRPM6 and TRPM7 in osteoblast-like cells under Mg^{2+}- and/or Ca^{2+}-reduced culture condition.

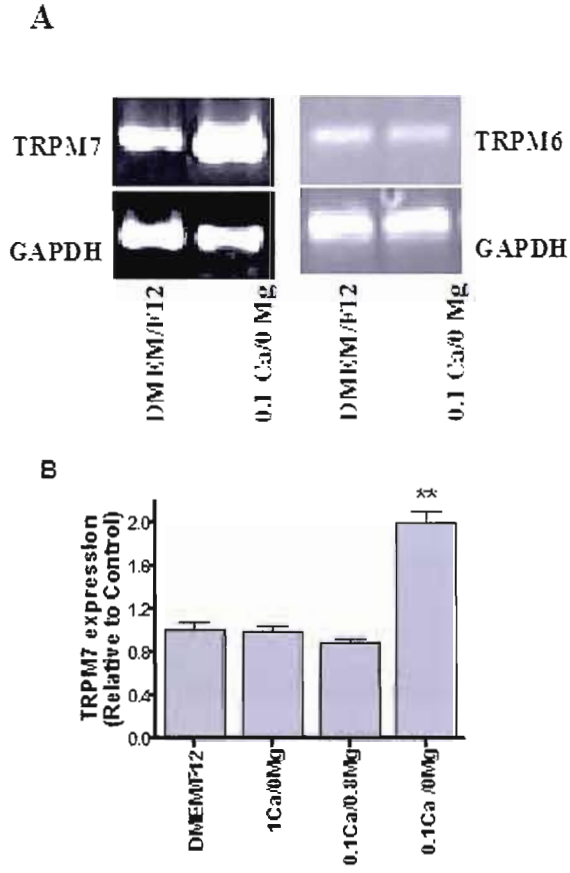


Fig. 6. Activation of TRPM7 under low extracellular Mg^{2+} levels.

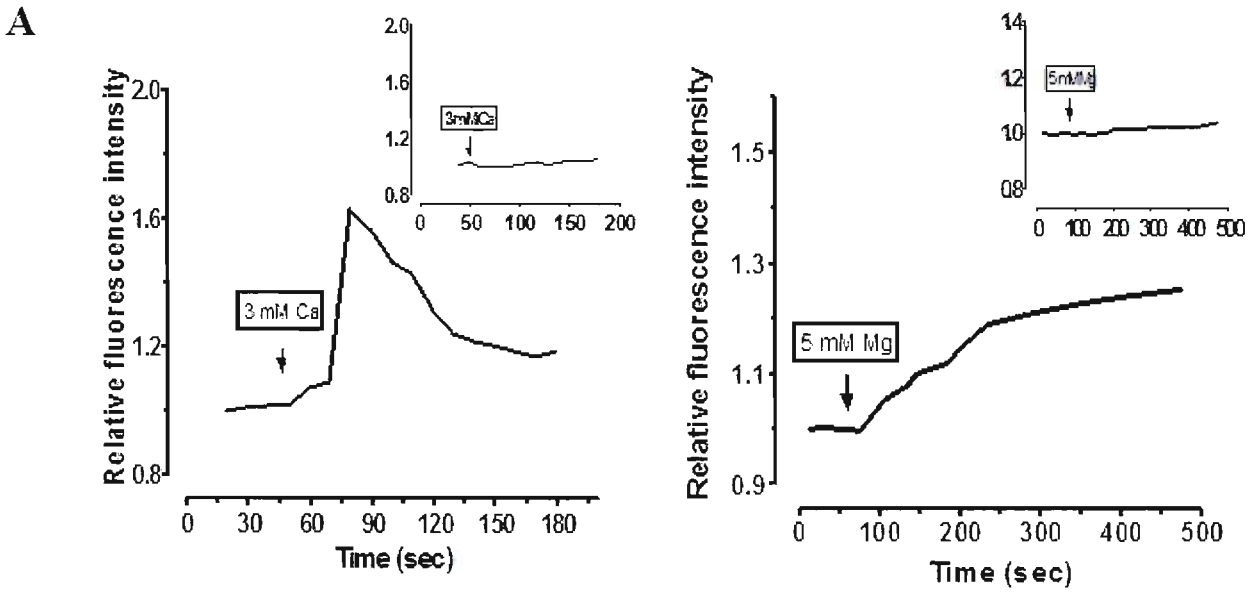


Fig. 6. Activation of TRPM7 under low extracellular Mg^{2+} levels

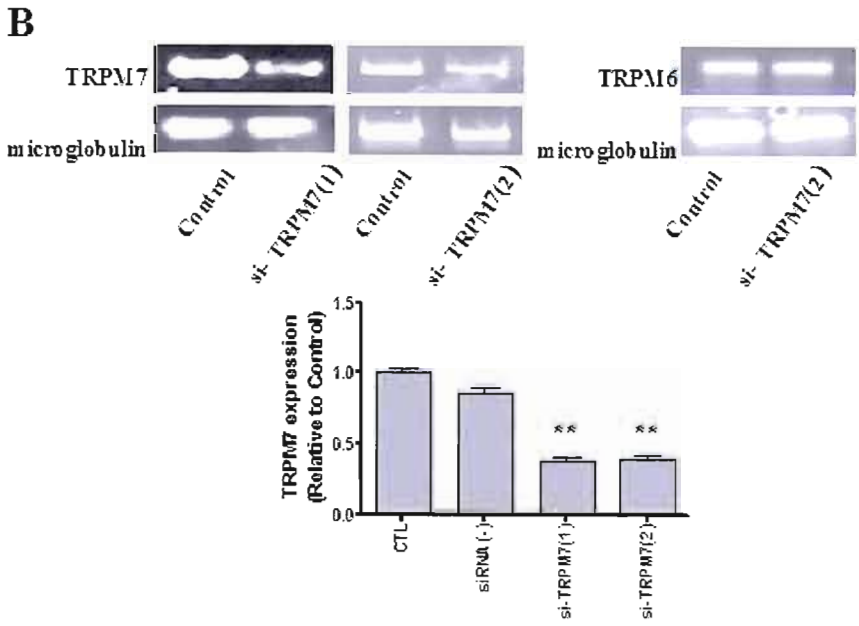


Fig. 6. Activation of TRPM7 under low extracellular Mg^{2+} levels.

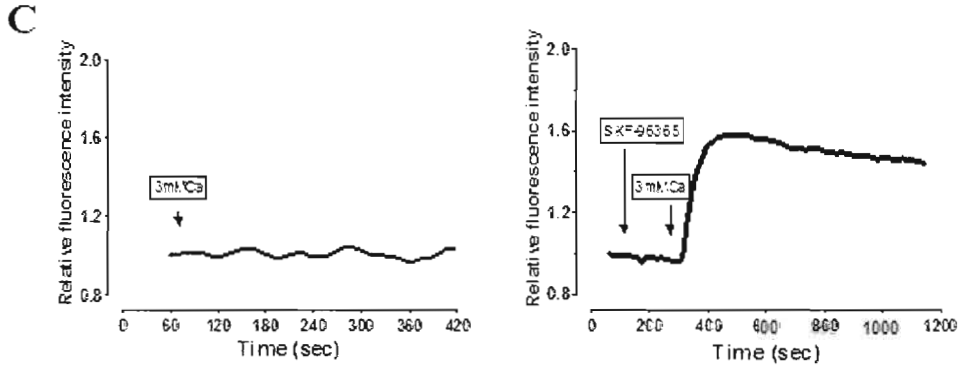
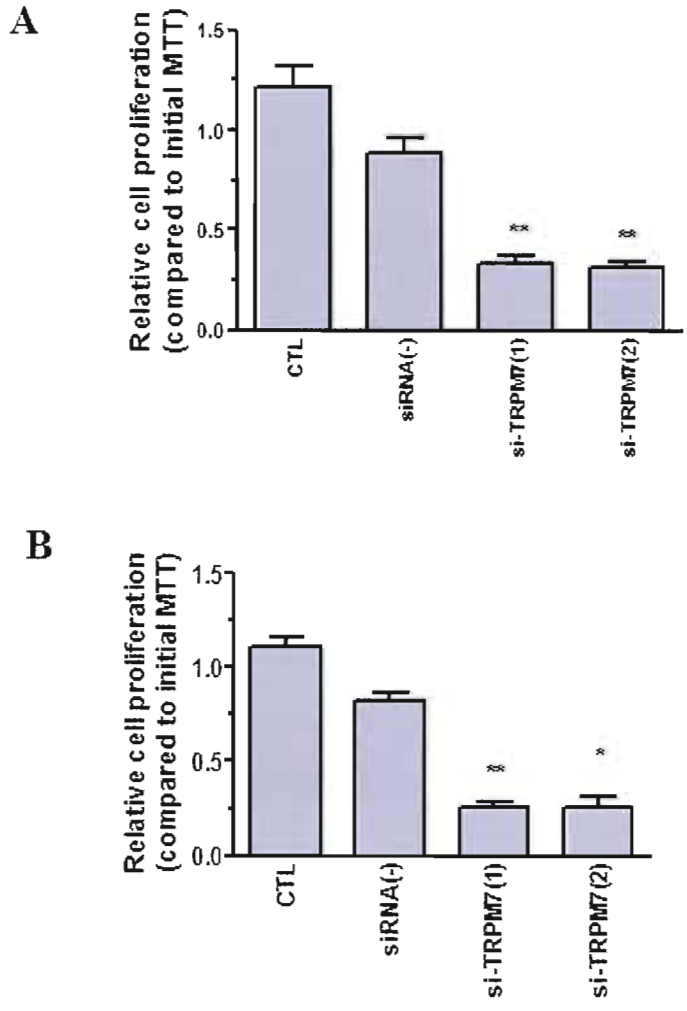


Fig. 7. Effect of reducing TRPM7 expression on osteoblast-like cell proliferation



2.3. Article 3 : Effet du magnésium et implication des canaux "melastatin-related transient receptor potential 7" (TRPM7) dans la prolifération et la migration des ostéoblastes induites par le « platelet-derived growth factor » (PDGF).

L'os est un tissu très dynamique en perpétuel renouvellement (désigné remodelage osseux), qui se caractérise par un équilibre entre la résorption de la matrice minéralisée par les ostéoclastes et la formation d'un nouveau tissu par les ostéoblastes. Cet équilibre dépend en grande partie de la prolifération et de la migration des ostéoblastes, et subséquemment de leur différenciation et de la sécrétion de protéines formant la matrice osseuse. Dans bien des cas où l'équilibre est perdu, il y a apparition d'ostéoporose (littéralement: la maladie des os poreux) qui est caractérisée par une masse osseuse réduite due à une dégradation osseuse supérieure à la formation osseuse, une fragilité osseuse et une susceptibilité accrue aux fractures. Parmi les facteurs de risque, une diète déficiente en magnésium (Mg) a été identifiée comme une condition prédisposant à une réduction graduelle de la masse osseuse et au développement de l'ostéoporose chez l'humain. Nos récents travaux indiquent que les canaux cationiques "melastatin related transient receptor potentiel" (TRPM7) assurent l'homéostasie du Mg intracellulaire, un ion important pour de nombreuses fonctions cellulaires. La présente étude visait à déterminer l'importance du Mg et des canaux TRPM7 dans la prolifération et la migration des ostéoblastes induites par le « platelet-derived growth factor » (PDGF), un facteur reconnu pour stimuler la prolifération et la migration des ostéoblastes lors du remodelage osseux. Des mesures de prolifération et de migration cellulaires ont indiqué qu'une réduction de Mg du milieu de culture diminue la prolifération et la migration des ostéoblastes stimulées par le PDGF. Ce dernier augmente l'expression du gène TRPM7 des ostéoblastes. De plus, une stratégie d'interférence à l'ARN ciblant TRPM7 réduit la prolifération et la migration des ostéoblastes induites par le PDGF. En conclusion, nos résultats indiquent que la stimulation de la prolifération et de la migration des ostéoblastes par le PDGF est favorisée par la présence de Mg extracellulaire et des canaux TRPM7. Ainsi, une déficience en Mg est à même de diminuer la formation osseuse et contribuer au développement de l'ostéoporose.

Cet article a été publié en 2009 dans « Am J Physiol Cell Physiol ». J'ai réalisé toutes les expériences dans ce manuscrit et rédigé le document sous la supervision de mon directeur de recherche qui a commenté et révisé le document.

Importance of Melastatin-like Transient Receptor Potential 7 and Magnesium in the Stimulation of Osteoblast Proliferation and Migration by Platelet-Derived Growth Factor

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Running title: Role of TRPM7 in PDGF-induced proliferation and migration of osteoblastic cells

Keywords: TRPM7 channels; osteoblasts; PDGF; proliferation; magnesium; migration; adhesion.

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Abstract

Bone is a dynamic tissue that is continuously being remodeled throughout life. Specialized cells called osteoclasts transiently break down old bone (resorption process) at multiple sites as other cells known as osteoblasts are replacing it with new tissue (bone formation). Usually, both resorption and formation processes are in balance and thereby maintain skeletal strength and integrity. This equilibrium is assured by the coordination of proliferation, migration, differentiation and secretory functions of the osteoblasts, which are essential for adequate formation and resorption processes. Disturbances of this equilibrium may lead to decreased bone mass (osteoporosis), increased bone fragility and susceptibility to fractures. Epidemiological studies have linked insufficient dietary magnesium (Mg^{2+}) intake in humans with low bone mass and osteoporosis. Here, we investigated the roles of Mg^{2+} and melastatin-like transient receptor potential 7 (TRPM7), known as Mg^{2+} channels, in human osteoblast cell proliferation and migration induced by platelet-derived growth factor (PDGF), which has been involved in the bone remodeling process. PDGF promoted an influx of Mg^{2+}, enhanced cell migration and stimulated the gene expression of TRPM7 channels in human osteoblast MG-63 cells. The stimulation of osteoblast proliferation and migration by PDGF was significantly reduced under culture conditions of low extracellular Mg^{2+} concentrations. Silencing TRPM7 expression in osteoblasts by specific siRNA prevented the induction by PDGF of Mg^{2+} influx, proliferation and migration. Our results indicate that

extracellular Mg^{2+} and TRPM7 are important for PDGF-induced proliferation and migration of human osteoblasts. Thus Mg^{2+} deficiency, a common condition among the general population, may be associated to altered osteoblast functions leading to inadequate bone formation and the development of osteoporosis.

Keywords: TRPM7 channels, osteoblast, proliferation, migration, PDGF, Magnesium.

Introduction

The bone is a dynamic tissue that is continuously being remodeled following two coordinated processes. The osteoclasts are constantly breaking down old bone (known as the resorption process) as the osteoblasts are replacing it with new tissue (termed the bone formation process). Osteoblasts ensure bone formation and tissue mineralization through the secretion of bone matrix components (type I collagen and noncollagenous proteins) and also provide factors essential for the differentiation of osteoclasts. By regulating osteoclast differentiation, the osteoblasts not only play a central role in bone formation, but also in the regulation of bone resorption (21). Therefore, the maintenance of bone remodeling equilibrium relies on the coordination of proliferation, migration, differentiation, secretion of matrix proteins and apoptosis of osteoblastic cells. Disturbances of any of these processes that shift the balance of equilibrium towards bone resorption may cause loss of bone mass with a consequent increase in bone fragility and susceptibility to fractures (osteoporosis).

Osteoblast cells arise from osteoprogenitor cells located in the bone marrow (2). Osteoprogenitors are induced to differentiate under the influence of growth factors, in particular the bone morphogenetic proteins (BMPs). Aside BMPs, other growth factors including fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) promote the division of osteoprogenitors and increase osteogenesis. It has been known that platelet-derived growth factor (PDGF) promotes proliferation and migration in a variety of cell types

including osteoblastic cells (23). This growth factor is one of the biological mediators of bone formation (5;17;29). Studies have shown that PDGF enhances DNA and collagen synthesis in rat osteoblast cultures (5) and increases bone matrix deposition in cultured calvaria (29). In an *in vivo* study, PDGF was also found to enhance bone formation (41).)

Calcium (Ca^{2+}) influx is implicated in numerous cellular functions such as proliferation, differentiation, secretion and apoptosis (3). In bone cells of the osteoblast lineage, calcium channels play fundamental roles in cellular responses to external stimuli including both mechanical forces and hormonal signals (11;18).

Magnesium (Mg^{2+}) is the most abundant intracellular divalent cation in living cells. Virtually every biological process requires Mg^{2+} as an essential cofactor for hundreds of enzymes, and for the regulation of various transporters and ion channels (35). Furthermore, Mg^{2+} is an important modulator of intracellular free Ca^{2+} concentration and intracellular pH which are major determinants of cell contraction, secretion, motility, and proliferation (10;28;46).

Epidemiologic studies have demonstrated a positive correlation between Mg^{2+} intake and bone density, thereby linking insufficient dietary Mg^{2+} intake to osteoporosis (for a review,(31)). By histomorphometry analysis, a reduction in osteoblast number was observed in Mg^{2+}-deficient rats and mice (33). In other studies, serum and bone alkaline phosphatase, osteocalcin and bone osteocalcin mRNA were reduced, suggesting a decrease in osteoblastic functions (4;9).

The 'membrane magnesium mitosis' model of cell proliferation control suggests that upon mitogenic stimulus, cells are able to increase their intracellular magnesium content, likely by activating Mg^{2+} influx, to levels optimum for the initiation of protein synthesis. Therefore, influx of both extracellular Ca^{2+} and Mg^{2+} for proper intracellular ion homeostasis is likely solicited for cell proliferation. Melastatin-like transient receptor potential (TRPM) is a recently emerging subfamily of the transient receptor potential family, a diverse group of voltage-independent Ca^{2+} -permeable cation channels expressed in mammalian cells (7;16;25;26) that encompasses eight distinct members, designated TRPM1–8. TRPM7 combines structural elements of both an ion channel and a protein kinase (for recent reviews, (12;15)). TRPM7 channels have been associated with cell proliferation and survival (27). TRPM7-deficient DT40 cells can be rescued from their cell growth defect by supplementary extracellular Mg^{2+} (36) suggesting a residual capacity of these cells to maintain intracellular Mg^{2+} homeostasis. Among TRPM members TRPM2, TRPM6 and TRPM7 uniquely possess an enzyme domain in their long C-termini, the latter two exhibiting spontaneously activated divalent cation (Ca^{2+}, Mg^{2+} and other trace metals) entry, regulated by cytosolic Mg^{2+} and ATP levels. Mutations in the TRPM6 gene have been shown in patients suffering from a hereditary form of hypomagnesaemia caused by impaired Mg^{2+} resorption (6). Life-long dietary Mg^{2+} supplementation of these patients is sufficient to rescue the phenotype of affected human beings. Our previous results have indicated that TRPM7 channels are involved in the intracellular Mg^{2+} homeostasis of osteoblastic cells as well as in basal cell proliferation (1).

To better understand the link between insufficient dietary Mg^{2+} intake in humans with low bone mass and osteoporosis, the current study aimed to investigate the importance of Mg^{2+} and TRPM7 channel in the stimulation of osteoblast proliferation and migration by the bone remodeling regulating factor PDGF.

Materials and methods

Cell culture

Human osteoblast-like MG-63 cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were grown in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (DMEM/F12; Sigma, Oakville, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Cansera, Etobicoke, Ontario, Canada), L-glutamine (Invitrogen, Burlington, Ontario, Canada) and penicillin/streptomycin (Invitrogen, Burlington, Ontario, Canada). Cells were cultured in 5% CO_2 at 37 °C and were harvested weekly with Trypsin-EDTA solution (Invitrogen, Burlington, Ontario, Canada).

Cell proliferation assays

For proliferation experiments, cells were seeded in 96-well plates (Sarstedt, Montréal, Québec, Canada) at 2500 cells/cm^2. After 4 days of culture in supplemented media, cells were incubated in calcium (Ca^{2+})- and Mg^{2+}-free DME/F12 (Sigma, Oakville, Ontario, Canada) supplemented with different concentrations of Ca^{2+} and Mg^{2+} without serum in the absence or the presence of 25 ng/ml of PDGF-BB (Sigma) for 48 h. Cell proliferation was determined by microtiter tetrazolium (MTT) reduction assays. Briefly, one hour before the end of treatment the medium was replaced with DMEM/F12 containing 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) (Sigma, Oakville, Ontario, Canada). At the end of the incubation, media was aspired and formazan crystals generated by the cellular

reduction activity were dissolved in dimetylsulfoxide (DMSO). Absorbance was measured at 575 nm and data are expressed as the ratio of absorbance of treated cells versus initial MTT absorbance corresponding to the level of MTT reduced to formazan crystals by cells, the initial day of treatment.

Cell migration assays

To investigate the effects of Ca^{2+}, Mg^{2+}, PDGF and the involvement of TRPM7 on MG-63 cell migration, a wound scratch assay was performed. Briefly, the cells were grown to confluent monolayer on 35-mm diameter dishes (Sarstedt). Monolayers were wounded by scratching the surface as uniformly as possible with a pipette tip and then cells were incubated for 18 h under different conditions. This initial wounding and the movement of the cells in the scratched area were photographically monitored using an Axiovert Zeiss 200 microscope with a 10X (0.25 numerical aperture) objective linked to a Coolsnap Es CCD camera. The 18h-time interval has been chosen because it is shorter than MG-63 doubling time in these conditions. Four different fields from each sample were analysed unsing the ImageJ software for quantitative estimations of the number of cells that have migrated to the wounded area. The values are expressed as the relative cell migration compared to control condition.

Adhesion assay

Cell adhesion was measured using a trypsinization assay (36). Briefly, cells were seeded in 12 well plates for 4 days and treated with different conditions for 16 h.

Then cells were incubated with 1 ml of 0.05% trypsin-EDTA for 4 min to promote cell detachment. The enzymatic activity was stopped by the addition of 1 ml of culture medium containing 10% FBS and detached cells were collected. The number of detached cells under limited trypsinization was then manually counted using a hemocytometer. The remaining adherent cells was collected by complete trypsinization and counted by hemocytometer. The percentage of adhesion was expressed as a ratio of detached versus total (detached and remaining cells) cells.

Cell morphology

MG-63 cells were cultured in 4-wells Labtek (Nalge Nunc, Napperville, IL, USA) dishes for 5 days in supplemented medium. Cells were then incubated for 18 h under different conditions. At the end of treatment, cells were fixed in 3.7% formaldehyde/phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% Triton X-100/PBS for 3 min and blocked in 3% bovine serum albumin/PBS. Cells were incubated with 6μM of Alexia fluor 555-conjugated phalloidin (1:200; Invitrogen) with an equivalent volume of 20% Pluronic F127 (Invitrogen) for 45 min at room temperature in the dark to detect filamentous actin (F-actin). Cells were washed three times with PBS between each step. The cells were examined with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300) with an Apochromatic 40X N.A. 1.0 objective lens. Fluorescence was excited by an argon laser at 555nm and emission was collected with a 565 filter.

Reverse-transcription polymerase chain reaction

Total RNA from cells was extracted using TriZol (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) reactions were carried out with Omniscript RT kit (Qiagen, Mississauga, Ontario, Canada) using hexamers. The PCR amplifications were conducted with *Taq* PCR core kit (Qiagen) using specific primer sets for human TRPM7 (sense: 5'-TGCACCTATACTAGGAAACGTTTTCG-3'; antisense: 5'-CATGATAAAAGGCATAAAACTT

TCGC-3'). Each primer was designed in distinct exons to ensure specific transcript amplifications. Briefly, amplifications were carried out for 40 cycles according to incubation of 1 min at 94°C, 30 sec at 58 °C and 1 min at 72°C. Amplification products were resolved in 2% agarose gel with ethidium bromide revelation.

Measurements of intracellular calcium and magnesium levels

MG-63 cells were cultured in 4-wells Labtek (Nalge Nunc) for 5 days in supplemented media. Cells were then transferred to HEPES-buffered saline solution (HBSS (mM): 121 NaCl, 5.4 KCl, 25 HEPES, 1.8 $CaCl_2$ and 6.0 $NaHCO_3$ at pH 7.3) or Ca^{2+}-free and/or Mg^{2+}-free solution (HBSS without Ca^{2+} and/or without Mg^{2+}) and loaded with 2μM Fluo-3 AM or Magnesium Green (Invitrogen) with an equivalent volume of 20% Pluronic F127 (Invitrogen) for 45 min at room temperature in the dark. Thereafter cells were washed with corresponding HBSS and the loaded dye was allowed to deesterify for 45 min at room temperature in the dark. Following transfer to a Ca^{2+}-free and Mg^{2+}-free HBSS, additions of Ca^{2+} or Mg^{2+} were made in an open

chamber configuration at room temperature. The cells were examined with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300) with an Apochromatic 40X N.A. 1.0 objective lens. Fluorescence was excited by an argon laser at 488nm and emission was collected with a 515nm filter. Data were analyzed with Laser Sharp 2.1T, Time Course 1.0 software for 7 to 8 fields per experiments (between 10 and 20 cells per field). All experiments were performed for at least four individual experiments.

Interference with siRNA

Small interfering RNAs directed against human TRPM7 (si-TRPM7) and a nontargeting control (si-mock) were obtained from Qiagen. Transfection of the siRNAs was performed using HiPerFect reagent following the manufacturer's instructions. Quantifications of transcripts were performed by RT-PCR 2 days post-transfection to evaluate the TRPM7 expression level as described previously (1). To investigate the role of TRPM7 in osteoblast proliferation, cells seeded in 96-well plates were transfected for 24 h, and thereafter incubated in appropriate conditions for 48 h with the addition of MTT for the last 1 h of incubation. For the determination of the importance of TRPM7 in migration, adhesion and morphology, cells seeded in 12-well plates were transfected for 48 h, and thereafter incubated in appropriate conditions for 18 h and studied as mentioned above. To study the involvement of TRPM7 in magnesium influx, cells seeded in 4-wells Labtek (Nalge Nunc) were transfected for 48 h and studied as mentioned above.

Statistical analysis

A non-parametric Mann-Whitney test was used to compare two independent groups of sampled data or a two-way ANOVA with Bonferroni post-test was used to test the effectives of two independent variables using GraphPad Prism 4. A level of $P<0.05$ was considered significant.

Results

The physiological concentration of Mg^{2+} is between 0.8 to 1.0 mM and concentrations ranging between 0.6 and 0.7 mM are frequent in apparently healthy western people with lowest levels of 0.4 to 0.5 mM in critically ill subjects (43). In experimental Mg^{2+} deficiency, plasma Mg^{2+} level of 0.1 mM has been observed in animal models (32). Mg^{2+} depletion has been shown to influence endothelial cell functions in a dose- and time-dependent manner (22). Such alterations were associated with modulations of gene expression related to cell adaptation. Based on these findings, we utilized experimental conditions of severe Mg^{2+} depletion for short period of time to principally document the roles of intracellular Mg^{2+} and TRPM7 activity on morphology, adhesion, cell migration and exclude cell adaptation to low Mg^{2+} concentration condition as reported by Maier (22).

Influence of reduced extracellular magnesium and calcium on the osteoblast proliferation induced by PDGF.

Influx of both extracellular Ca^{2+} and Mg^{2+} for proper intracellular ion homeostasis is likely solicited for cell proliferation. We determined the importance of extracellular Ca^{2+} and Mg^{2+} in the basal proliferation of osteoblastic cells and induced by PDGF. MTT assays were performed on MG-63 cells maintained for 48 h in 1 mM Ca^{2+}- and Mg^{2+}-free media supplemented with various concentrations of Mg^{2+} ions (Fig. 1A) or in 0.8 mM Mg^{2+} and Ca^{2+}-free media supplemented with various concentrations of

Ca^{2+} ions (Fig. 1B). As shown in Fig. 1A, concentrations of Mg^{2+} below 0.8 mM reduced gradually basal cell proliferation compared to control condition. Moreover, the stimulation of cell proliferation by PDGF was significantly abolished by concentrations of Mg^{2+} below 0.01 mM. As shown in Fig. 1B, no difference of cell proliferation was observed for concentration of Ca^{2+} below 1 mM in the absence or in the presence of PDGF. Similar reduction of cell proliferation under extracellular low Mg^{2+} conditions was obtained with two other human osteoblast-like cells, namely SaOs and U2OS (data not shown).

Influence of reduced extracellular magnesium and calcium on the osteoblast migration induced by PDGF.

A previous study showed that PDGF induces human osteoblast cell migration (13). We therefore sought to determine the involvement of Mg^{2+} or Ca^{2+} in basal or induced cell migration by PDGF. As shown in Fig. 1C, treatment with low Mg^{2+} (0.1mM) concentrations reduced the basal migration of MG-63 cells, while cell migration was not affected in low Ca^{2+} (0.1mM) medium. In addition we observed that the PDGF treatment stimulated MG-63 cell migration, which was reduced by decreasing Mg^{2+} concentration in the media. Again, low Ca^{2+} (0.1mM) concentration had no effect on the migration of MG-63 induced by PDGF. These results indicate the importance of Mg^{2+} in the stimulation of MG-63 migration by PDGF.

Importance of calcium and magnesium in cell morphology and adhesion.

Migration phenomena implicate changes in the organization of cytoskeleton elements which correlate with changes in cell morphology and adhesion cells (38). Therefore,

we investigated the role of extracellular Ca^{2+} and Mg^{2+} in osteoblast morphology and adhesion in the absence or presence of PDGF. As shown in fig 2A, the morphology of cells after 18h of treatment in low Ca^{2+} (0.1mM) condition medium was similar to CTL (1mMCa^{2+} and 0.8mM Mg^{2+}) condition but when treated in low Mg^{2+} (0.1mM) condition, cell morphology was different compared to CTL condition and cells showed fewer projections (right panels show greater magnification). Similar results were obtained for cells treated with PDGF. We undertook to correlate this difference with a reorganization of actins filaments of cytoskeleton. Indeed in Fig 2B, phalloidin staining for F-actin of cells incubated for 18h in low Ca^{2+} (0.1mM) condition was similar to control condition but when cells were treated in low Mg^{2+} (0.1mM) condition, phalloidin staining showed less and more diffuse actin staining. PDGF stimulation revealed more actin-rich and fingerlike projections (arrows) extending from the membrane compared to control condition. Similar effects on F-actin were observed in low Mg^{2+} (0.1mM) or low Ca^{2+} (0.1mM) conditions in the presence of PDGF (data not shown). Furthermore, as shown in Fig. 2C, incubation of cells for 18 h under low Mg^{2+} condition reduced both basal cell adhesion and induced by PDGF compared to control condition, whereas reduction of extracellular Ca^{2+} has no effect on basal adhesion and induced by PDGF.**Effect of PDGF on the influx of calcium and magnesium.**

Our results indicate that extracellular magnesium is important for PDGF-induced human osteoblast cell proliferation and migration. In order to investigate the importance of Mg^{2+} and Ca^{2+} influx in the stimulation of cell proliferation and

migration by PDGF, we performed intracellular Mg^{2+} and Ca^{2+} measurements with Magnesium Green and Fluo3 respectively. As shown in Fig. 3A, the addition of PDGF to cells following prior incubation in 1 mM Ca^{2+} and Mg^{2+}-free medium had no effect on the intracellular Mg^{2+} level of MG-63 cells in the absence of extracellular Mg^{2+}. On the other hand, prior incubation of cells in 1 mM Ca^{2+} and Mg^{2+}-free medium resulted in the observation of a Mg^{2+} influx upon addition of PDGF to the incubation medium in the presence of extracellular 0.8 mM Mg^{2+} (Fig. 3B) suggesting that PDGF activated plasma membrane Mg^{2+} channels. Under pre-incubation conditions of the Fig. 3A, the addition of PDGF to cells in 0.8mM Mg^{2+}, Ca^{2+}-free media resulted in a mobilization of Ca^{2+} from intracellular stores (Fig. 3C). To note, although mobilization of Ca^{2+} was induced by PDGF, fluorescence of Magnesium green was not modified as shown in fig3A, indicating that the increase of Magnesium Green fluorescence was specific to Mg^{2+} influx.

Effect of PDGF on the expression of TRPM7 by osteoblast MG-63.

Our results indicate that PDGF promotes the activation of plasma membrane channels that ensure Mg^{2+} influx. Our previous studies have shown that TRPM7 channels ensure homeostasis of Mg^{2+} influx in MG-63 cells (1). Therefore, we speculated that TRPM7 channels may be important in the effects of PDGF on osteoblastic cells. First, we investigated the effect of PDGF on the expression levels of TRPM7 channels. As shown in Fig. 4, the expression of TRPM7 increased by approximately 2-fold after a 4h-treatment with PDGF compared to control condition where cells had received fresh medium. This up-regulation of TRPM7 expression was sustained for a 24 h-

period. On the other hand, no difference was noticed for the expression of TRPM6 (data not shown). Our results suggest that PDGF up regulates TRPM7 to ensure long term Mg^{2+} homeostasis in osteoblastic cells which promotes proliferation, and migration in osteoblastic cells.

Effect of PDGF on cell proliferation, migration, adhesion and Mg influx under condition of reduced TRPM7 expression.

In order to determine the importance of TRPM7 channels in the proliferation of osteoblastic cells, MTT assays were performed with cells transfected with specific siRNA against TRPM7 or non-targeted siRNA. The Fig. 5A shows that the basal and PDGF-induced proliferations of MG-63 cells were inhibited by 60-75% under conditions where TRPM7 expression was reduced with specific siRNAs against TRPM7 (right panel). Moreover, cell migration (fig. 5B) and adhesion (fig. 6B) induced by PDGF were prevented when cells were transfected with siRNA against TRPM7 compared to mock condition without PDGF. We also observed upon reduction of TRPM7 expression that the morphology of cells was different to CTL and mock conditions with less projection and the shape of cells was similar to low Mg^{2+} condition (Fig. 6A). This difference was correlated to a reorganization of actins filaments. Fluorescence intensity of Alexia fluor 555-conjugated phalloidin in cells silenced for the expression of TRPM7 was reduced and more diffused compared to CTL and mock conditions where actin-rich and fingerlike projections (arrows) were easily distinguishable. Furthermore, the reduction of TRPM7 expression prevented Mg^{2+} influx induced by PDGF (fig. 6C) even when high concentrations of Mg^{2+} (10

mM) were added in the incubation medium (data not shown), which suggest the absence of compensation pathway for Mg^{2+} influx.

Discussion

As a reduction in osteoblast number and osteoporosis have been reported under insufficient dietary Mg^{2+} intake (33) and given that during bone remodeling osteoblast proliferation and migration are stimulated in response to growth factors such as PDGF (24), we investigated the importance of Mg^{2+} and of TRPM7, a channel known for its involvement in the homeostasis of intracellular Mg^{2+} (1), in the stimulation of osteoblast proliferation and migration by PDGF. Our study indicates that appropriate extracellular Mg^{2+} levels are essential to the stimulation of osteoblast proliferation and migration by PDGF. Moreover, TRPM7 channels appear a key player in the Mg^{2+} influx induced by PDGF. Therefore, we provide mechanistic evidence for the alterations of osteoblast functions associated with insufficient dietary Mg^{2+} intakes which are susceptible to lead to the development of osteoporosis.

It has been shown that PDGF can promote proliferation and migration in a variety of cell types including osteoblastic cells (23). In accordance, we observed that PDGF stimulates proliferation and migration of human osteoblast-like MG-63 cells. Of interest, we observed a significant reduction of osteoblast proliferation and migration induced by PDGF under culture conditions of low extracellular Mg^{2+} (0.1mM), while conditions with low extracellular Ca^{2+} (0.1mM) concentrations were without effect. These results highlight the important role of Mg^{2+} in osteoblast functions. The observation that the stimulation of osteoblast proliferation by PDGF was reduced under low extracellular Mg^{2+} conditions agrees with our previous report about the

importance of this ion for osteoblast proliferation (1). Of interest in the current study is that Mg^{2+} also influences the migration of osteoblastic cells as low extracellular concentrations reduced both basal and induced cell migration. Migration phenomena implicate changes in the organization of cytoskeleton elements which is associated with modifications in cellular morphology and adhesion (38). So we further investigated the effects of low concentrations of extracellular Mg^{2+} (0.1mM) or Ca^{2+} (0.1mM) on cell morphology, adhesion and F-actin organization We observed that basal cell adhesion and induced by PDGF were reduced by low extracellular Mg^{2+} (0.1mM) whereas the reduction of extracellular Ca^{2+} (0.1mM) has no effect on basal cell adhesion or induced by PDGF. A change of cell morphology (fewer projections) and reorganization of actin filaments were also observed under low Mg^{2+} (0.1mM) conditions; similar effects were observed for low Mg^{2+}(0.1mM) condition in the presence of PDGF while low Ca^{2+} (0.1mM) condition have similar shape compared to control condition even when they were treated with PDGF. Thereby our results highlight the importance of Mg^{2+} in maintaining the structural integrity of osteoblastic cells, a necessary parameter for numerous cellular functions (30). Overall, these results indicate that Mg^{2+} is required for the effects of PDGF on proliferation, adhesion and migration of osteoblastic cells. Extracellular Mg^{2+} has been associated with integrin mediated cell adhesion of tumorigenic osteoblastic cells to type I collagen while this ion was without influence on cell adhesion to laminin-l and fibronectin (44). However, it is not yet clear whether the importance of Mg^{2+} in cell adhesion relies on intracellular, extracellular or both levels. We speculated that the

effects of PDGF on cell proliferation, morphology, adhesion and migration rely on adequate intracellular Mg^{2+} homeostasis. So we hypothesized that PDGF may promote Mg^{2+} influx to induce cell proliferation and migration in human osteoblasts. Indeed, Mg^{2+} influx were observed upon the addition of PDGF to the incubation medium indicating that plasma membrane channels were activated. Our previous results have indicated that TRPM7 channel is involved in the intracellular Mg homeostasis of osteoblastic cells (1). In accordance, PDGF may activate TRPM7 channels to assure Mg^{2+} influx. Indeed, the silencing of TRPM7 expression prevented the Mg^{2+} influx induced by PDGF while siRNA treatments had no effect on the depletion of intracellular Ca^{2+} stores induced by PDGF. Moreover, we showed that the expression of TRPM7 is up-regulated by treatment with PDGF. The enhanced expression of TRPM7 was specific since the expression of TRPM6, a close homologue of TRPM7, was not modified by PDGF. This growth factor may promote up-regulation of TRPM7 expression to ensure long term Mg^{2+} homeostasis in osteoblastic cells. Of interest silencing TRPM7 expression, in addition to prevent PDGF-induced Mg^{2+} influx, inhibited the stimulation of cell proliferation, and migration by PDGF. A change of cell morphology (fewer projections) was also observed when TRPM7 expression was reduced by specific siRNA which correlate with our previous observations under low extracellular Mg^{2+} (0.1mM) conditions. Therefore, our results suggest that TRPM7-mediated Mg^{2+} influx is important for PDGF-induced cell proliferation and migration in human osteoblasts. Such Mg^{2+} influx will likely ensure the adequate intracellular ion levels essential for numerous

cellular functions triggered by PDGF. To note, no Mg influx was seen in TRPM7-deficient cells stimulated by PDGF even high 10 mM Mg^{2+} concentrations were added to the incubation medium, which suggest the absence of compensation pathway for Mg^{2+} influx. Our previous published results also indicate that no other Mg^{2+} entry pathway can supply for TRPM7 silencing in the osteoblasts (1). In accordance, studies have reported both rescue (19;27;37) and no effect (14;45) by Mg^{2+} supplementation in TRPM7-deficient cells which has been stated to depend on cell types. More specifically, over-expression of other Mg^{2+} entry pathways, such as the Mg^{2+} transporter SLC41A2, has been shown to compensate for the loss of TRPM7 channel and maintain Mg^{2+} at sufficient levels for the cells to proliferate in a manner which directly correlates with their level of expression (34).

In a molecular point of view, it has been reported that TRPM7 channels regulate cell adhesion through m-calpain (protease that control cell adhesion through focal adhesion disassembly) by mediating the local influx of calcium into peripheral adhesion complexes (39). The cellular adhesion and motility were enhanced when the expression levels of TRPM7 were reduced. In contrast, Clark and al (8) have demonstrated in rat basophilic leukemia (RBL) cells that the C-terminal alpha kinase activity of TRPM7 channels regulates myosin IIA filament stability and localization by phosphorylating a short stretch of amino acids within the α-helical tail of the myosin IIA heavy chain. They observed that low over expression of TRPM7 increased induces cell spreading and increases cell adhesion. Sugden and al (40) have suggested that both Ca^{2+} and Mg^{2+} can bind tightly to myosin, although only the

binding of Mg^{2+} shows a direct influence on the enzymatic activity. On the other hand, Mg^{2+} is an important modulator of the intracellular Ca^{2+} levels, the latter being associated with cell contraction, proliferation, migration, and secretion (42). Therefore, changes in intracellular Mg^{2+} levels could lead to significant effects on Ca^{2+}-dependent signaling pathways that regulate cellular functions.

Magnesium deficiency is common among the general population as its intake has decreased over the years in a significant proportion of the population, especially in the western world. Many important human pathologies such as hypertension, heart failure, several nervous system complaints, muscle diseases and atherosclerosis have been associated with a decrease in Mg^{2+} availability (20). In addition, coexisting disorders that impair intestinal Mg^{2+} absorption and/or are associated with renal Mg^{2+} loss, such as malabsorption syndromes, alcoholism, diabetes mellitus and drugs (e.g. diuretics), would place an individual at even greater risk. Physiological concentration of Mg^{2+} is 0.8 to 1.0 mM and concentrations ranging between 0.6 and 0.7 mM are frequent in apparently healthy western people with lowest levels of 0.4 to 0.5 mM in critically ill subjects (43). Of interest, epidemiologic studies provide a link associating insufficient dietary Mg^{2+} intake in humans with low bone mass and osteoporosis (for a review, see (31)). Moreover, genetic hypomagnesaemia with renal Mg^{2+} wasting leads to low bone mass (31). Experimental Mg^{2+} deficiency in animal models, where plasma Mg^{2+} levels range from 0.1 to 0.4 mM, has resulted in impaired bone growth, osteopaenia, and increased skeletal fragility with a decrease in both osteoblast number and osteoclast activity associated to a form of aplastic bone

disease (32;33). Progressive alterations of the endothelial cell functions, in accordance with a pro-atherogenic environment leading to cardiovascular disease, have been reported following 1 to 6 days of cell culture in low (0.1 and 0.5 mM) Mg^{2+} concentration conditions (22). Such alterations were associated with modulations of gene expression related to cell adaptation. Therefore, Mg^{2+} depletion influences cell functions in a dose- and time-dependent manner. Our experimental protocol corresponds to severe Mg^{2+} depletion conditions for short period of time that may not directly apply to human disease which is more likely associated with moderate Mg^{2+} deficiency for long period of time, as years. However, such experimental strategy has the benefit to exclude potential cell adaptation to low Mg^{2+} concentration condition as reported by Maier (22) and allow investigating the roles of intracellular Mg^{2+} and TRPM7 activity on cell morphology, adhesion, proliferation and migration. Our current results indicate that Mg^{2+} influx and TRPM7 are important for PDGF-induced human osteoblast proliferation and migration. Such reduction in osteoblast cell proliferation would lead to inadequate bone formation and poor regulation of resorption, resulting in the development of osteoporosis.

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References

1. **Abed E, Moreau R.** Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation. *Cell Prolif* 40: 849-865, 2007.

2. **Aubin JE.** Advances in the osteoblast lineage. *Biochem Cell Biol* 76: 899-910, 1998.

3. **Berridge MJ, Lipp P, Bootman MD.** The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1: 11-21, 2000.

4. **Carpenter TO, Mackowiak SJ, Troiano N, Gundberg CM.** Osteocalcin and its message: relationship to bone histology in magnesium-deprived rats. *Am J Physiol* 263: E107-E114, 1992.

5. **Centrella M, McCarthy TL, Canalis E.** Platelet-derived growth factor enhances deoxyribonucleic acid and collagen synthesis in osteoblast-enriched cultures from fetal rat parietal bone. *Endocrinology* 125: 13-19, 1989.

6. **Chubanov V,Waldegger S, Schnitzler M, Vitzthum H, Sassen MC, Seyberth HW, Konrad M, Gudermann T.** Disruption of TRPM6/TRPM7 complex formation by a mutation in the TRPM6 gene causes hypomagnesemia with secondary hypocalcemia. *Proc Natl Acad Sci USA* 101:2894-2899, 2004.

7. **Clapham DE, Runnels LW, Strubing C.** The TRP ion channel family. *Nat Rev Neurosci* 2: 387-396, 2001.

8. **Clark K, Langeslag M, van Leeuwen B, Ran L, Ryazanov AG, Figdor CG, Moolenaar WH, Jalink K, van Leeuwen FN.** TRPM7, a novel regulator of actomyosin contractility and cell adhesion. *EMBO J* 25: 290-301, 2006.

9. **Creedon A, Flynn A, Cashman K.** The effect of moderately and severely restricted dietary magnesium intakes on bone composition and bone metabolism in the rat. *Br J Nutr* 82: 63-71, 1999.

10. **D'Angelo EK, Singer HA, Rembold CM.** Magnesium relaxes arterial smooth muscle by decreasing intracellular Ca^{2+} without changing intracellular Mg^{2+}. *J Clin Invest* 89: 1988-1994, 1992.

11. **Duncan RL, Akanbi KA, Farach-Carson MC.** Calcium signals and calcium channels in osteoblastic cells. *Semin Nephrol* 18: 178-190, 1998.

12. **Fleig A, Penner R.** The TRPM ion channel subfamily: molecular, biophysical and functional features. *Trends Pharmacol Sci* 25: 633-639, 2004.

13. **Gilardetti RS, Chaibi MS, Stroumza J, Williams SR, Antoniades HN, Carnes DC, Graves DT.** High-affinity binding of PDGF-AA and PDGF-BB to normal human osteoblastic cells and modulation by interleukin-1. *Am J Physiol* 261: C980-C985, 1991.

14. **Hanano T, Hara Y, Shi J, Morita H, Umebayashi C, Mori E, Sumimoto H, Ito Y, Mori Y, Inoue R.** Involvement of TRPM7 in cell growth as a spontaneously activated Ca^{2+} entry pathway in human retinoblastoma cells. *J Pharmacol Sci* 95: 403-419, 2004.

15. **Harteneck C.** Function and pharmacology of TRPM cation channels. *Naunyn Schmiedebergs Arch Pharmacol* 371: 307-314, 2005.

16. **Harteneck C, Plant TD, Schultz G.** From worm to man: three subfamilies of TRP channels. *Trends Neurosci* 23: 159-166, 2000.

17. **Howes R, Bowness JM, Grotendorst GR, Martin GR, Reddi AH.** Platelet-derived growth factor enhances demineralized bone matrix-induced cartilage and bone formation. *Calcif Tissue Int* 42: 34-38, 1988.

18. **Iqbal J, Zaidi M.** Molecular regulation of mechanotransduction. *Biochem Biophys Res Commun* 328: 751-755, 2005.

19. **Kim BJ, Park EJ, Lee JH, Jeon JH, Kim SJ, So I.** Suppression of transient receptor potential melastatin 7 channel induces cell death in gastric cancer. *Cancer Sci* 99:2502-2509, 2008.

20. **Laires MJ, Monteiro CP, Bicho M.** Role of cellular magnesium in health and human disease. *Front Biosci* 9: 262-276, 2004.

21. **Mackie E.** Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell Biol* 35: 1301-1305, 2003.

22. **Maier JA, Malpuech-Brugere C, Zimowska W, Rayssiguier Y, Mazur A.** Low magnesium promotes endothelial cell dysfunction: implications for atherosclerosis, inflammation and thrombosis. *Biochim Biophys Acta* 1689: 13-21, 2004.

23. **Mehrotra M, Krane SM, Walters K, Pilbeam C.** Differential regulation of platelet-derived growth factor stimulated migration and proliferation in osteoblastic cells. *J Cell Biochem* 93: 741-752, 2004.

24. **Mehrotra M, Krane SM, Walters K, Pilbeam C.** Differential regulation of platelet-derived growth factor stimulated migration and proliferation in osteoblastic cells. *J Cell Biochem* 93: 741-752, 2004.

25. **Minke B, Cook B.** TRP channel proteins and signal transduction. *Physiol Rev* 82: 429-472, 2002.

26. **Montell C, Birnbaumer L, Flockerzi V.** The TRP channels, a remarkably functional family. *Cell* 108: 595-598, 2002.

27. **Nadler MJ et al.** LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. *Nature* 411: 590-595, 2001.

28. **Nakajima T, Iwasawa K, Hazama H, Asano M, Okuda Y, Omata M.** Extracellular Mg^{2+} inhibits receptor-mediated $Ca(^{2+})$-permeable non-selective cation currents in aortic smooth muscle cells. *Eur J Pharmacol* 320: 81-86, 1997.

29. **Pfeilschifter J, Oechsner M, Naumann A, Gronwald RG, Minne HW, Ziegler R.** Stimulation of bone matrix apposition in vitro by local growth factors: a comparison between insulin-like growth factor I, platelet-derived growth factor, and transforming growth factor beta. *Endocrinology* 127: 69-75, 1990.

30. **Rubin H.** The membrane, magnesium, mitosis (MMM) model of cell proliferation control. *Magnes Res* 18: 268-274, 2005.

31. **Rude RK, Gruber HE.** Magnesium deficiency and osteoporosis: animal and human observations. *J Nutr Biochem* 15: 710-716, 2004.

32. **Rude RK, Gruber HE, Norton HJ, Wei LY, Frausto A, Mills BG.** Bone loss induced by dietary magnesium reduction to 10% of the nutrient requirement in rats is associated with increased release of substance P and tumor necrosis factor-alpha. *J Nutr* 134: 79-85, 2004.

33. **Rude RK, Kirchen ME, Gruber HE, Meyer MH, Luck JS, Crawford DL.** Magnesium deficiency-induced osteoporosis in the rat: uncoupling of bone formation and bone resorption. *Magnes Res* 12: 257-267, 1999.

34. **Sahni J, Nelson B, Scharenberg AM.** SLC41A2 encodes a plasma-membrane Mg^{2+} transporter. *Biochem J* 401: 505-513, 2007.

35. **Schmitz C, Deason F, Perraud AL.** Molecular components of vertebrate Mg^{2+}-homeostasis regulation. *Magnes Res* 20: 6-18, 2007.

36. **Schmitz C, Dorovkov MV, Zhao X, Davenport BJ, Ryazanov AG, Perraud AL.** The channel kinases TRPM6 and TRPM7 are functionally nonredundant. *J Biol Chem* 280: 37763-37771, 2005.

37. **Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, Kurosaki T, Fleig A, Scharenberg AM.** Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. *Cell* 114: 191-200, 2003.

38. **Small JV, Kaverina I, Krylyshkina O, Rottner K.** Cytoskeleton cross-talk during cell motility. *FEBS Lett* 452: 96-99, 1999.

39. **Su LT, Agapito MA, Li M, Simonson WT, Huttenlocher A, Habas R, Yue L, Runnels LW.** TRPM7 regulates cell adhesion by controlling the calcium-dependent protease calpain. *J Biol Chem* 281: 11260-11270, 2006.

40. **Sugden EA, Nihei T.** The effects of calcium and magnesium ions on the adenosine triphosphatase and inosine triphosphatase activities of myosin A. *Biochem J* 113: 821-827, 1969.

41. **Tanaka H, Wakisaka A, Ogasa H, Kawai S, Liang CT.** Effect of IGF-I and PDGF administered in vivo on the expression of osteoblast-related genes in old rats. *J Endocrinol* 174: 63-70, 2002.

42. **Touyz RM.** Transient receptor potential melastatin 6 and 7 channels, magnesium transport, and vascular biology: implications in hypertension. *Am J Physiol Heart Circ Physiol* 294: H1103-H1118, 2008.

43. **Verive MJ, Irazuzta J, Steinhart CM, Orlowski JP, Jaimovich DG.** Evaluating the frequency rate of hypomagnesemia in critically ill pediatric patients by using multiple regression analysis and a computer-based neural network. *Crit Care Med* 28: 3534-3539, 2000.

44. **Vihinen P, Riikonen T, Laine A, Heino J.** Integrin alpha 2 beta 1 in tumorigenic human osteosarcoma cell lines regulates cell adhesion, migration, and invasion by interaction with type I collagen. *Cell Growth Differ* 7: 439-447, 1996.

45. **Wykes RC, Lee M, Duffy SM, Yang W, Seward EP, Bradding P.** Functional transient receptor potential melastatin 7 channels are critical for human mast cell survival. *J Immunol* 179: 4045-4052, 2007.

46. **Zhang A, Cheng TP, Altura BM.** Magnesium regulates intracellular free ionized calcium concentration and cell geometry in vascular smooth muscle cells. *Biochim Biophys Acta* 1134: 25-29, 1992.

Legend of the figures

Fig. 1. **Effect of extracellular Ca^{2+} and Mg^{2+}on MG-63 proliferation and migration.** Cells were incubated for 48 h in culture media containing (A) normal 1 mM Ca^{2+} and increasing concentrations of Mg^{2+} or (B) normal 0.8 mM Mg^{2+} and increasing concentrations of Ca^{2+} in the absence or the presence of 25 ng/ml PDGF-BB. Cell proliferation was determined by MTT assays and expressed as the means ± SEM of the ratio of the absorbance compared to initial MTT (MTT_i) of 4 individual experiments. Mann-Whitney test: $*P < 0.03$ compared to control condition without PDGF, $^{\Phi}P < 0.03$ compared to control condition with PDGF (CTL: 1 mM Ca^{2+} and 0.8 mM Mg^{2+}). C) Cells were grown to confluent monolayer, wounded by scratching the surface and incubated for 18 h in culture media containing 1 mM Ca^{2+} and 0.8 mM Mg^{2+} (CTL), 1 mM Ca^{2+} and 0.1 mM Mg (low Mg^{2+}) or 0.1 mM Ca^{2+} and 0.8 mM Mg^{2+} (low Ca^{2+}), all media was treated without or with PDGF (25ng/ml final concentration). The initial wounding (0 h) and the movement of the cells in the scratched area after 18 h were photographically monitored. The number of cells that have migrated to the wounded area was determined using ImageJ software. Values are expressed as means ± SEM of the relative cell migration of 4 independent experiments. Mann-Whitney test: $*P < 0.03$, $^{\#}P<0.03$ compared with the control condition without PDGF, $^{\Phi}P<0.03$ compared with the control condition with PDGF.

Fig. 2. **Effect of extracellular Ca^{2+} and Mg^{2+}on MG-63 morphology and adhesion induced by PDGF.** Cells were incubated for 18 h in culture media containing 1 mM Ca^{2+} and 0.8 mM Mg (CTL), 1 mM Ca^{2+} and 0.1 mM Mg (low Mg^{2+}) or 0.1 mM Ca^{2+} and 0.8 mM Mg (low Ca^{2+}) without or with 25 ng/ml PDGF. A) Cell morphology was photographically monitored after 18h. B) The actin cytoskeleton was stained using Alexia fluor 555-conjugated phalloidin and F-actin was visualized by fluorescence microscopy. C) The determination of cell adhesion was performed by trypsinisation as described in Materials and methods section and compared to control condition. Data are means ± SEM of at least 5 individual experiments. Mann-Whitney test: $^{*}P < 0.03$ compared with the control condition (left panel) and $^{\Phi}P < 0.03$ compared with the control condition with PDGF (right panel).

Fig. 3. **Effect of PDGF on Mg^{2+} and Ca^{2+} influx.** Cells were loaded for a 2h-incubation period with Magnesium Green in HBSS with 1mM Ca^{2+} without Mg^{2+} (A, B) or Fluo3-AM in HBSS without Ca^{2+} with 0.8 mM Mg^{2+} (C). Thereafter cells were transferred to HBSS: A) with 1 mM Ca^{2+} without Mg^{2+}, (B) with 1 mM Ca^{2+} and 0.8 mM Mg^{2+} or C) without Ca^{2+} with 0.8 mM Mg^{2+}, and then PDGF (25ng/ml final concentration) were added to the incubation medium. Measurements of intracellular Mg^{2+} or Ca^{2+} were performed with a laser scanning confocal as described in the Material and Methods section. All experiments were performed for at least four individual experiments with analyses of 7 to 8 fields per experiment (between 10 and 20 cells per field).

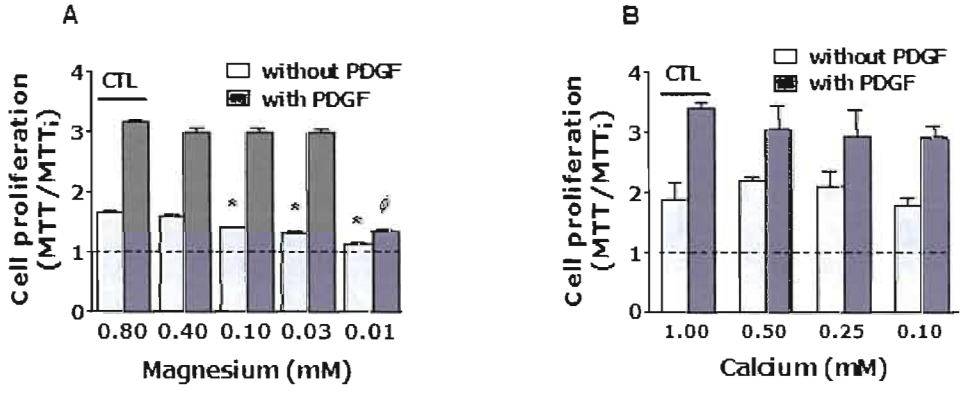
Fig. 4. **Effect of PDGF on the expression of TRPM7 by osteoblast MG-63.** A) Cells were incubated for 4, 16 and 24 h in fresh medium without or with PDGF. Total RNA was isolated from CTL cells (0 h) prior to treatments and from cells cultured under previous conditions. The levels of TRPM7 transcripts were determined by semi-quantitative RT-PCR as described in Material and Methods. The relative levels of TRPM7 expression compared to condition without PDGF are expressed as the mean ± SEM of 3 independent experiments. Two-way ANOVA. Bonferroni post tests: $***P < 0.001$ compared with the condition without PDGF.

Fig. 5. **Effect of reducing TRPM7 expression on osteoblast-like cell proliferation (A) and migration (B) induced by PDGF.** Cells were transfected with specific siRNAs against human TRPM7 (si-TRPM7) or with nontargeting control siRNA (si-Mock). The relative expression of human TRPM7 in MG-63 cells was determined after 48 h by RT-PCR and normalized according to the expression of GAPDH. A) Cell proliferation was determined after 48 h and compared to the MTT activity following 48-h transfection with nontargeting control siRNA (si-Mock) or with si-TRPM7 in the absence or the presence of 25 ng/ml PDGF. Data are expressed as the means ± SEM of 4 individual experiments and expressed as the ratio of the absorbance compared to initial MTT (MTTi). Mann-Whitney test: $*P < 0.03$ compared to si-Mock condition, $^{\Phi}P < 0.03$ compared to si-Mock condition with PDGF. B) Cells were grown to confluent monolayer and were wounded by scratching the surface. This initial wounding (0 h) and the movement of the cells in the scratched area after 18 h were photographically monitored. The number of cells that have migrated to the

wounded area was determined using ImageJ software. Values are expressed as means ± SE of 5 independent experiments. Mann-Whitney test: $*P < 0.03$, significant decrease compared to siRNA-Mock condition with PDGF

Fig. 6. **Effect of reducing TRPM7 expression on MG-63 morphology, adhesion and Mg influx induced by PDGF.** Cells were transfected with si-TRPM7 or si-Mock as mentioned above for 48 h. (A) Cell morphology was photographically monitored after 18h. B) Measurements of cell adhesion without stimulation by PDGF after 18h were performed as described in Materials and Methods section and compared to mock condition. Data are means ± SEM of at least 4 individual experiments. Mann-Whitney test: $*P < 0.03$ compared with the si-Mock condition. C) Cells were transfected with si-TRPM7 or si-Mock for 48 h and were loaded for a 2h incubation period with Magnesium Green in HBSS with 1mM Ca^{2+} without Mg^{2+}. Thereafter, cells were transferred to HBSS with with 1mM Ca^{2+} and 0.8mM Mg^{2+} and then PDGF concentration (25ng/ml final concentration) were added to the incubation medium. Measurements of intracellular Mg^{2+} were performed as described in the Material and Methods section. Data are means ± SEM of three to four individual experiments with analyses of 7 to 8 fields per experiments (between 10 and 20 cells per field). Mann-Whitney test: $*P < 0.03$ compared with the si-Mock condition. (D) Cells were transfected with si-TRPM7 or si-Mock (without PDGF) as mentioned above for 48 h and the actin cytoskeleton were stained using Alexia fluor 555-conjugated phalloidin and F-actin was visualized by fluorescence microscopy.

Fig. 1. Effect of extracellular Ca^{2+} and Mg^{2+} on MG-63 proliferation and migration.



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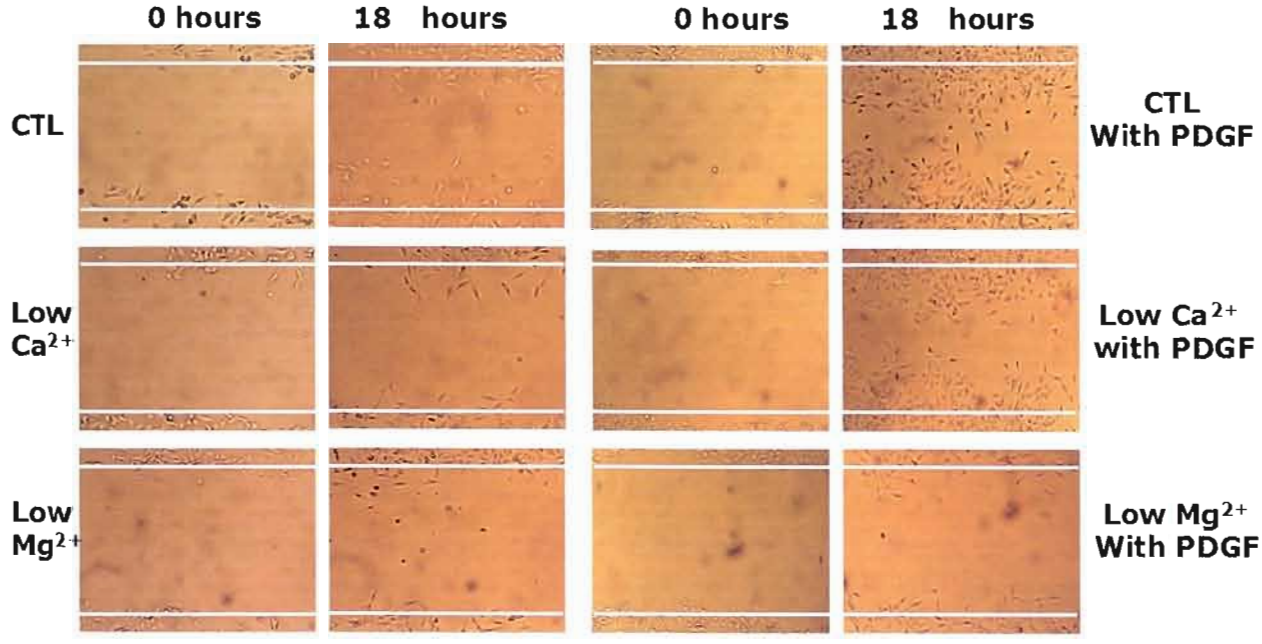


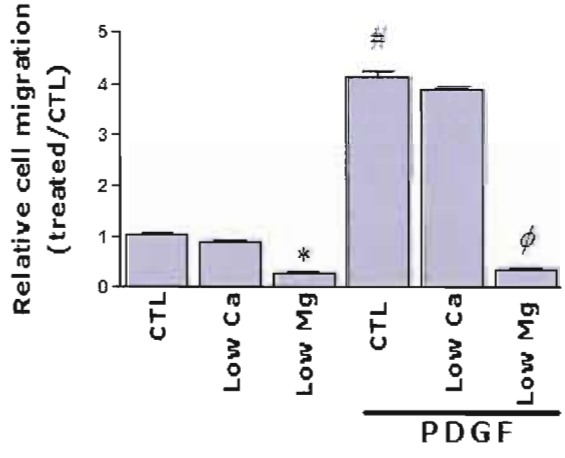


Fig. 2. Effect of extracellular Ca^{2+} and Mg^{2+}on MG-63 morphology and adhesion induced by PDGF

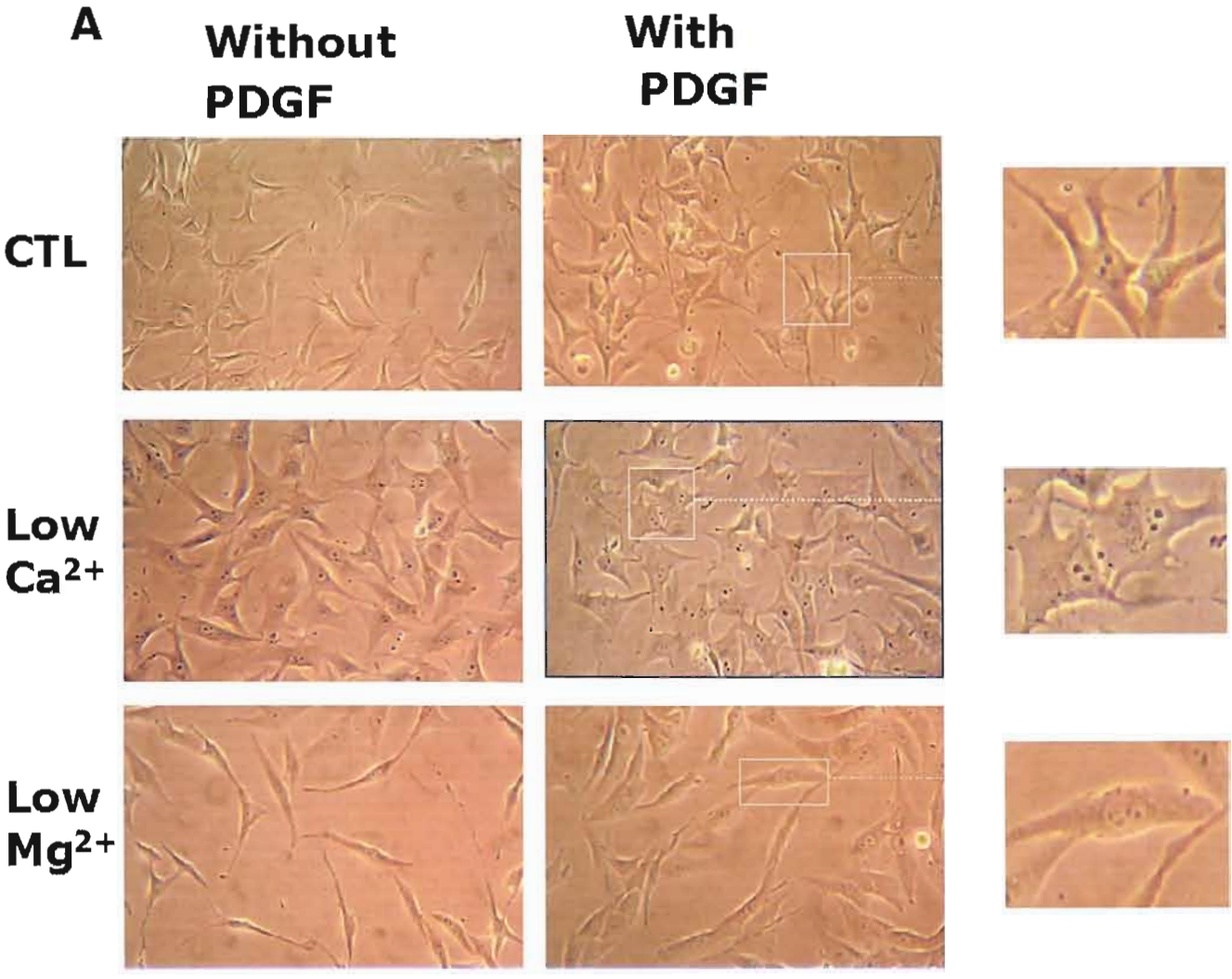
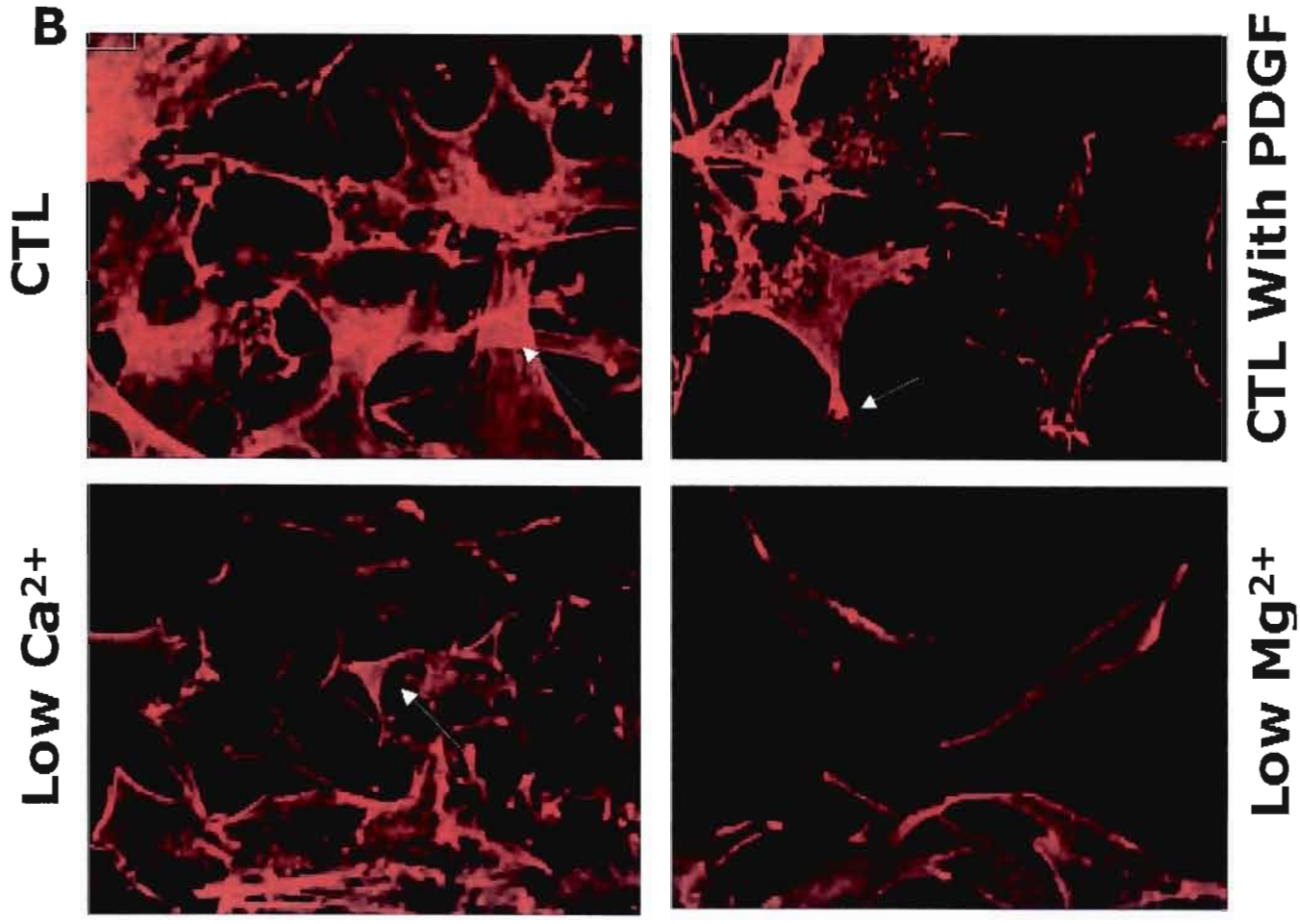


Fig. 2. Effect of extracellular Ca^{2+} and Mg^{2+}on MG-63 morphology and adhesion induced by PDGF



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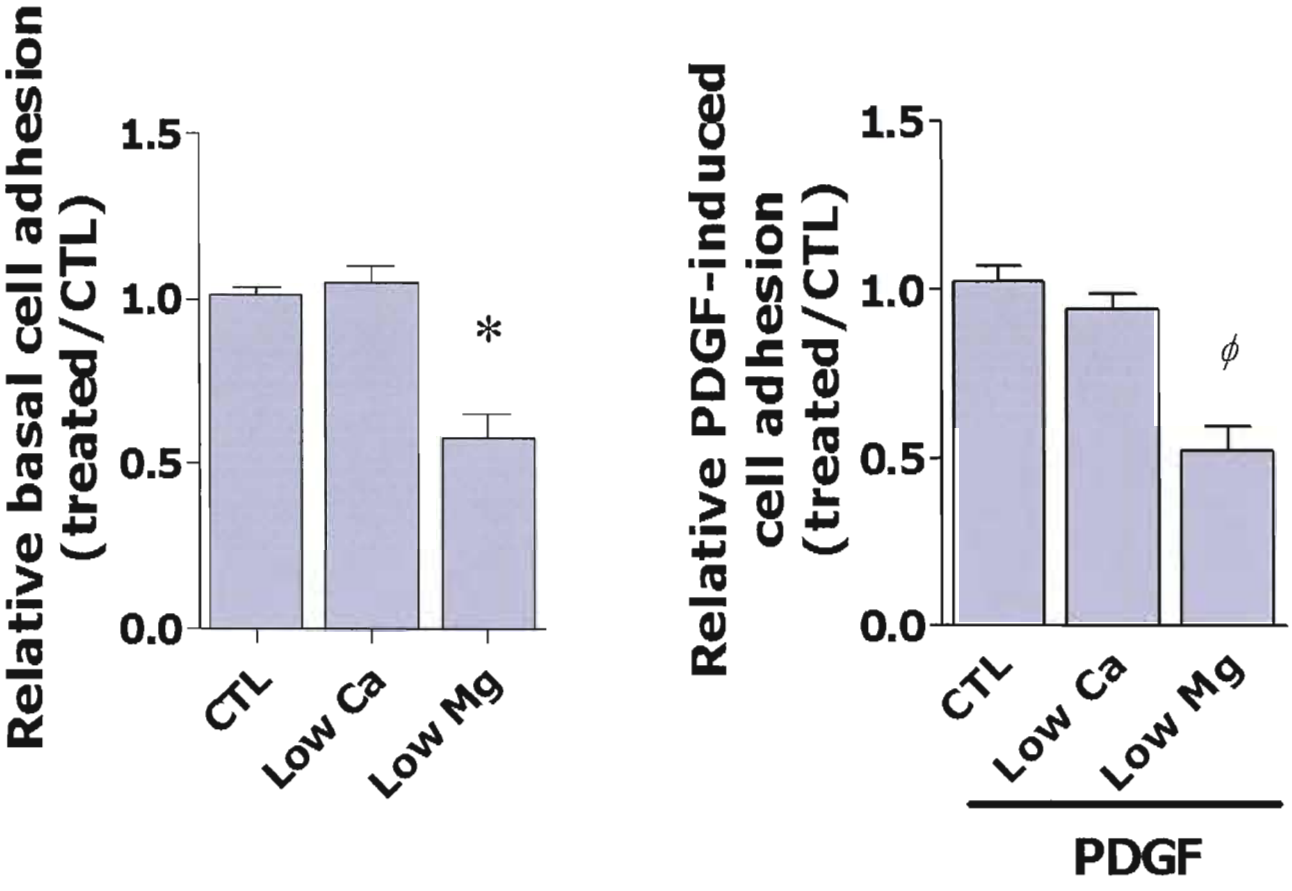


Fig. 3. Effect of PDGF on Mg^{2+} and Ca^{2+} influx

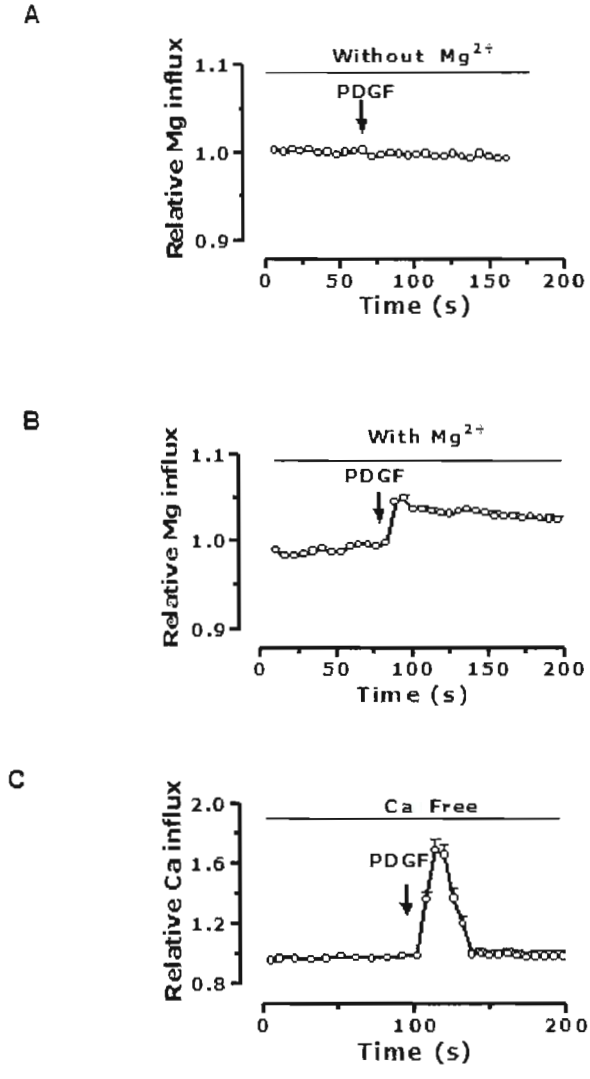


Fig. 4. Effect of PDGF on the expression of TRPM7 by osteoblast MG-63.

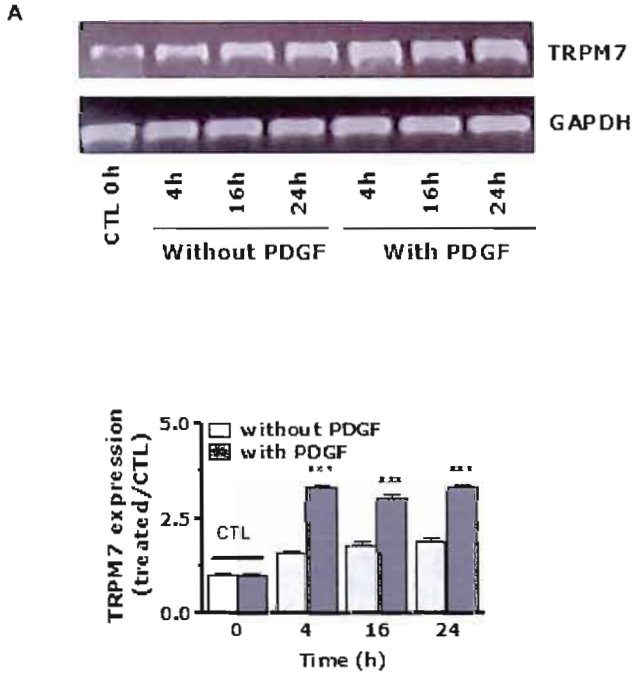
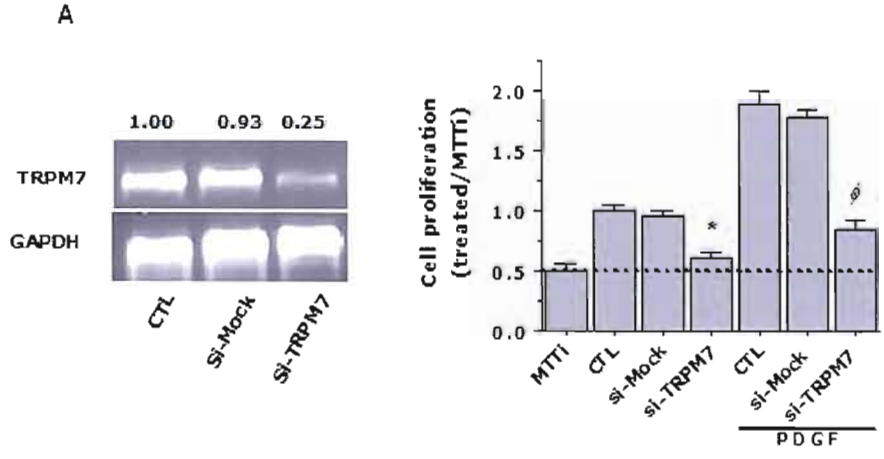


Fig. 5. Effect of reducing TRPM7 expression on osteoblast-like cell proliferation (A) and migration (B) induced by PDGF



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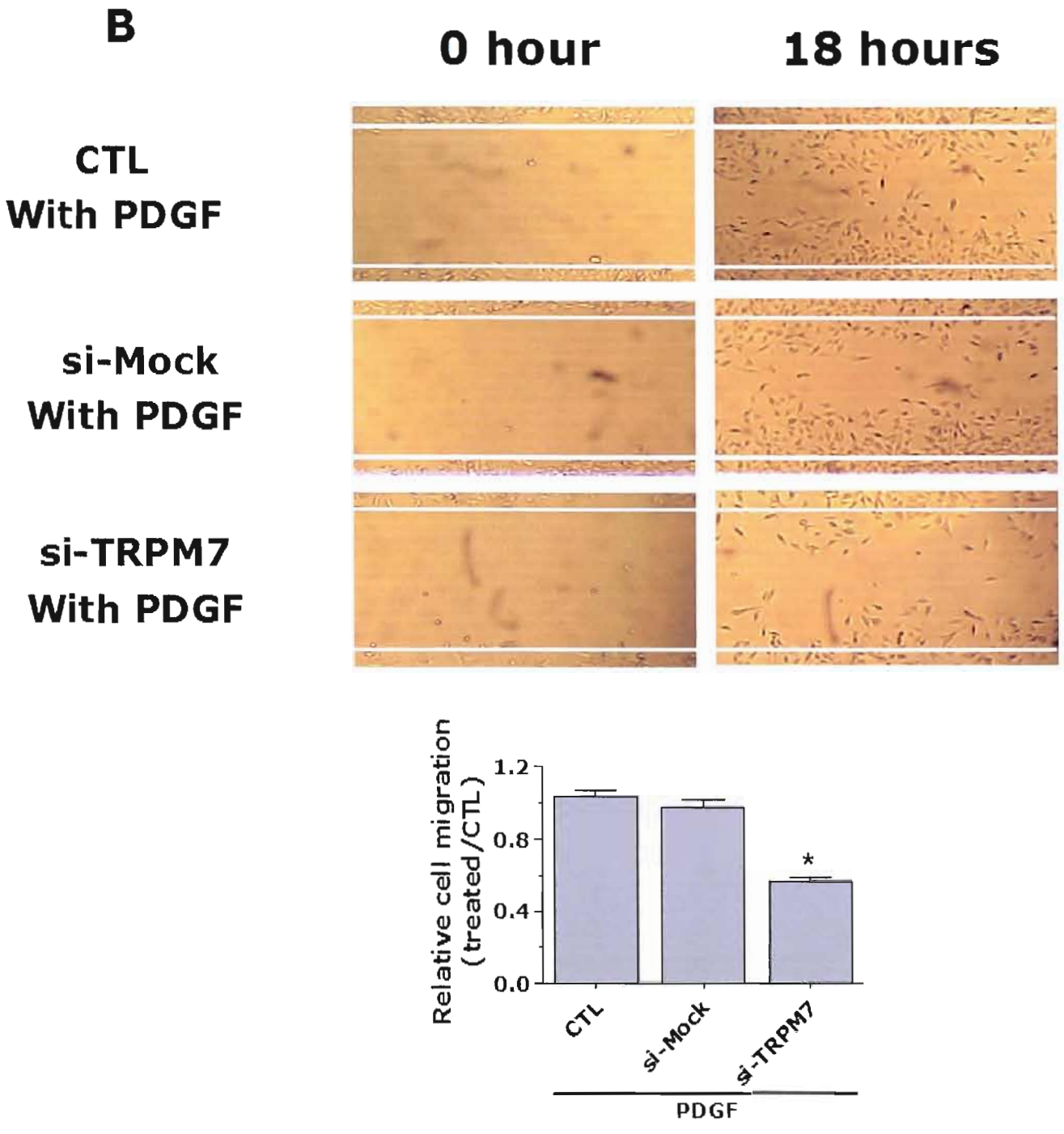
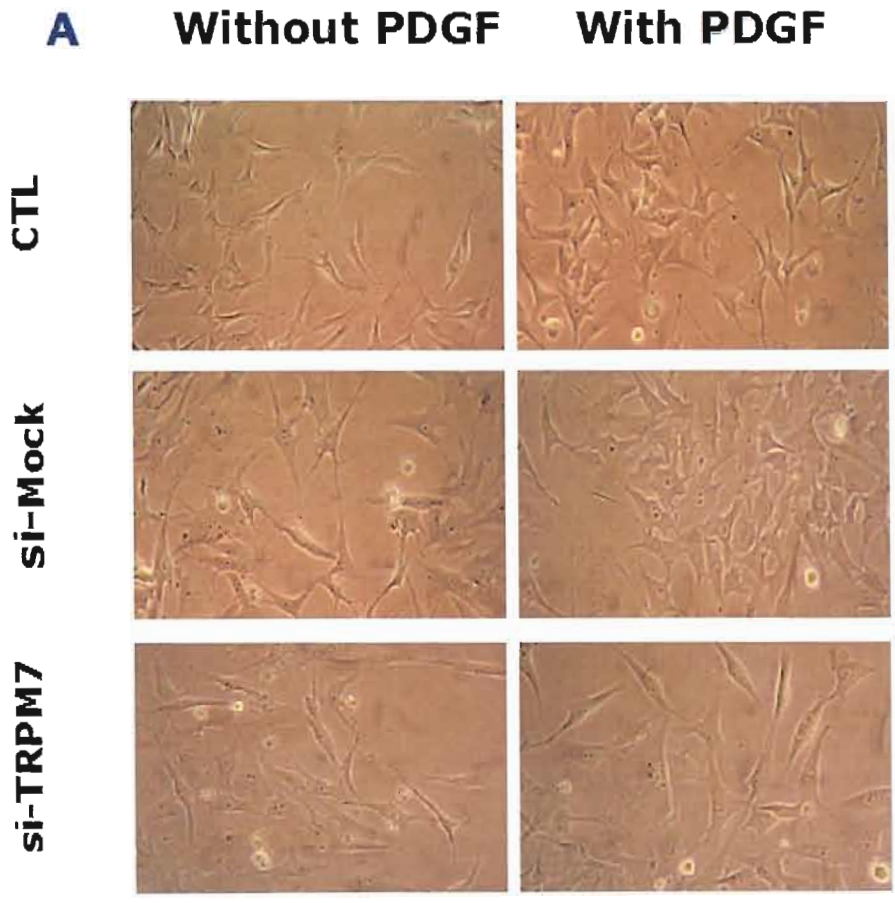


Fig. 6. Effect of reducing TRPM7 expression on MG-63 morphology, adhesion and Mg influx induced by PDGF.



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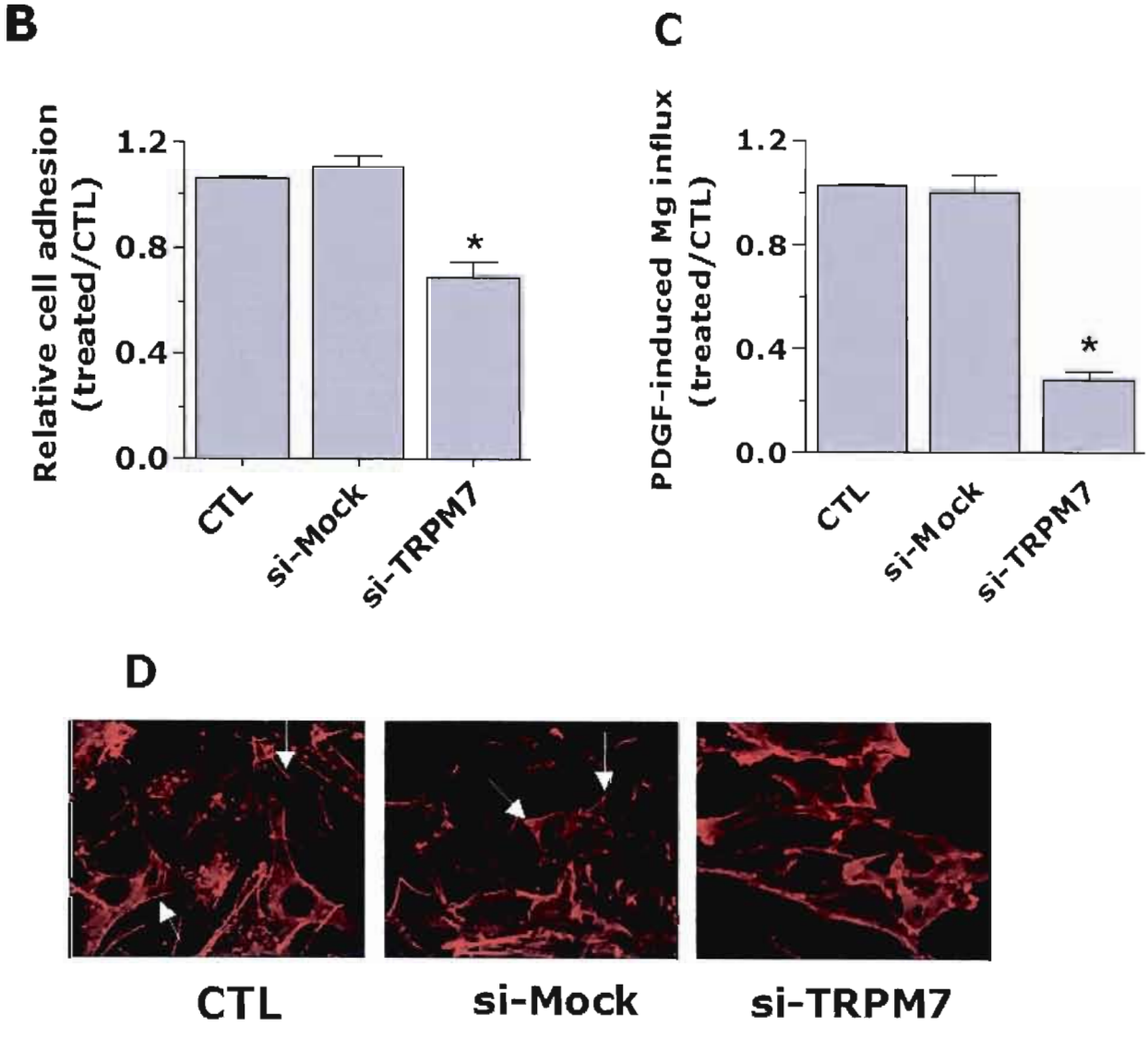

2.4. Article 4 : Effets du magnésium et implication des canaux cationiques TRPM7 dans la différenciation des cellules ostéoprogénitrices MC3T3.

L'ostéoporose est une maladie très fréquente qui se caractérise par une masse osseuse réduite due à une dégradation de la matrice minéralisée par les ostéoclastes supérieure à la formation osseuse par les ostéoblastes. Parmi les facteurs de risque, une diète déficiente en magnésium (Mg) a été identifiée comme une condition prédisposant à une réduction graduelle de la masse osseuse et au développement de l'ostéoporose chez l'humain. Nos récents travaux indiquent que les canaux «melastatin related transient receptor potentiel 7» (TRPM7) assurent l'homéostasie du Mg intracellulaire des ostéoblastes, un ion important pour de nombreuses fonctions cellulaires. La présente étude visait à déterminer l'importance des canaux TRPM7 dans la différenciation des ostéoprogéniteurs MC3T3 en ostéoblastes matures et au niveau de leur capacité à déposer et minéraliser la matrice osseuse. L'analyse des marqueurs de différenciation des ostéoblastes (ostéocalcine, phosphatase alcaline) et les mesures de minéralisation (rouge alizarine) ont indiqué qu'une réduction de Mg du milieu de culture diminue la capacité des cellules MC3T3 à se différencier en ostéoblastes matures et à déposer une matrice osseuse minéralisée. De plus, les cellules MC3T3 en voie de différenciation exprimaient davantage le gène TRPM7. Par ailleurs, une stratégie d'interférence à l'ARN ciblant TRPM7 a réduit la capacité des MC3T3 à se différencier, à déposer et à minéraliser la matrice osseuse. En conclusion, nos résultats indiquent que la différenciation des cellules ostéoprogénitrices MC3T3 en ostéoblastes matures est favorisée par la présence de Mg extracellulaire et l'expression des canaux TRPM7. Ainsi, une diète déficience en Mg est à même de diminuer la formation osseuse et contribuer au développement de l'ostéoporose.

Cet article est en révision pour le journal « Journal of Cellular Biochemistry ». J'ai réalisé toutes les expériences dans ce manuscrit et rédigé le document sous la supervision de mon directeur de recherche qui a commenté et révisé le document. J'aimerais mentionner la participation de Corine Martineau, étudiante à la maîtrise au laboratoire, pour la mise au point du modèle de différenciation.

Role of TRPM7 channels in the osteoblastic differentiation of murine MC3T3 cells

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Abstract

Adequate bone formation is assured by coordinated proliferation, migration, differentiation and secretory functions of the osteoblasts. Epidemiological studies have provided a link associating insufficient dietary magnesium intake with low bone mass and osteoporosis. Here, we investigated the role of melastatin-like transient receptor potential 7 (TRPM7), a calcium and magnesium channel, in the osteoblastic differentiation of murine MC3T3 cells. Cell differentiation was assessed by the increase of osteoblast markers such as osteocalcin, alkaline phosphatase activity and mineralization. Gene expression of TRPM7 increased with osteoblastic differentiation. Low extracellular calcium or magnesium conditions of culture significantly reduced the osteoblastic differentiation of MC3T3 cells. Silencing TRPM7 expression during the differentiation period also reduced osteoblastic differentiation. Our results indicate that TRPM7 are important for the differentiation of MC3T3. Thus magnesium deficiency, a common condition among the population, may be associated to altered osteoblastic differentiation leading to inadequate bone formation and the development of osteoporosis.

Keywords: TRPM7 channels, osteoblast, differentiation, magnesium.

Introduction

The bone is a dynamic tissue that is continuously being remodelled throughout life by two cell types: the osteoclasts which are constantly breaking down old bone (known as the resorption process) and the osteoblasts that are replacing it with new tissue (termed the bone formation process) [1]. The bone remodelling equilibrium which ensure bone mass maintenance relies on the coordinated proliferation, migration, differentiation, secretion of matrix proteins and apoptosis rate of osteoblastic cells [2]. Disturbances of any of these processes that shift the balance of equilibrium towards bone resorption may cause loss of bone mass with a consequent increase in bone fragility and susceptibility to fracture (osteoporosis) [3].

Epidemiologic studies have demonstrated a correlation between Mg intake and bone density, thereby linking insufficient dietary Mg intake to osteoporosis (for a review,[4]). Mg deficiency has been associated with impaired bone growth, reduced bone formation, increased bone resorption, osteoporosis, and increased skeletal fragility [5, 6]. By histomorphometry analysis, a reduction in osteoblast number was observed in Mg-deficient rats and mice [7]. In other studies, Mg deprivation was associated with reduced levels of serum bone alkaline phosphatase and osteocalcin, suggesting a decrease in osteoblastic differentiation [8, 9].

Osteoblast cells arise from osteoprogenitor cells located in the bone marrow [10]. Osteoprogenitors are induced to differentiate to mature osteoblast under the influence of growth factors, in particular the bone morphogenetic proteins (BMPs) and

regulated by multiple endocrine, paracrine, and autocrine factors. During differentiation, osteoblast cells express various phenotypic markers such as high alkaline phosphatase (ALP) activity and synthesize collagenous and noncollagenous bone matrix proteins including osteocalcin that makes up the extracellular matrix (ECM) which eventually starts to mineralize. Initiation of mineralization requires the precipitation and attachment of calcium phosphate crystals to the ECM, although the exact mechanism is poorly understood [8]. Osteoblastic differentiation from mesenchymal progenitor cells, secretion and mineralization of the extracellular matrix (ECM) are important steps of bone formation.

Our previous results have indicated that adequate extracellular Mg is important in basal and platelet-derived growth factor (PDGF)-induced osteoblast proliferation and migration. Indeed, culture conditions of low Mg levels and PDGF promote TRPM7 activation to ensure intracellular Mg homeostasis in osteoblasts [11, 12]. Moreover, low Mg and Ca levels and PDGF up-regulate gene expression of TRPM7 [11, 12]. Silencing TRPM7 gene expression prevents basal and platelet-derived growth factor (PDGF)-induced osteoblast proliferation [11] and migration [12]. Since bone formation also relies on osteoblastic differentiation and secretion of matrix proteins, we formulated the hypothesis that extracellular Mg and TRPM7 may influence osteoblast differentiation and mineralisation of matrix extracellular (ECM).

To better understand the link between insufficient dietary Mg intake in humans with low bone mass and osteoporosis, the current study aimed to investigate the

importance of Mg and TRPM7 channels in the osteoblastic differentiation of murine MC3T3 cells.

Materials and methods

Cell culture. MC3T3-E1 cells (American Type Culture Collection, ATCC; Rockville, MD, USA) derived from mouse calvaria constitute a well-known *in vitro* model for studying osteoblastic differentiation process [13]. MC3T3 cells were maintained in alpha-modified minimum essential medium (alpha-MEM; Sigma, Oakville, Ontario, Canada) and subcultured every 7 days with 0.05% trypsin-0.02% EDTA solution (Invitrogen, Burlington, Ontario, Canada) as described previously [14].

Osteoblastic differentiation. At confluence (designated D0), generally 7 days post-seeding at a 15 000 cells/cm^2 density in 24-well plates (Starstedt, Montréal, Quebec, Canada), cells were cultured during an additional 28-days culture period in a basal MEM medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Cansera, Etobicoke, Ontario, Canada) (condition designated D28B) or induced to differentiate by incubation in a MEM medium containing 10% FBS, 10 mM β-glycerophosphate (Sigma) and 50 µg/mL ascorbic acid (Sigma) during an additional 28-days culture period (designated D28T). To study the role of Ca and Mg on osteoblast differentiation, incubations were performed in Ca- and Mg-free DME/F12 (Sigma) with the addition of appropriate ion concentrations.

Alkaline phosphatase activity. Cells grown in 24-well plates (Starstedt) were solubilised in ice-cold buffer (100 mM glycine, 1 mM $MgCl_2$, 0.5% Triton X-100, pH 10.5) for alkaline phosphatase (ALP) activity determination by conversion of *p*-nitrophenylphosphate (*p*-NPP; Sigma) into *p*-nitrophenolate (*p*-NP) as described

previously [11]. ALP activity was normalized to the total protein content of the cell lysate. Protein concentrations were quantified by MicroBCA protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard.

Quantitative Alizarin Red Staining. Mineralized bone-like nodules were estimated following incubation of wells with an Alizarin Red S solution (ARS : 40 mM in a 10% (w/v) cetylpyridinium chloride [CPC] buffer dissolved in 10 mM Na_2PO_4, pH 4.1; all from Sigma) at room temperature under agitation. Wells were washed 3 times with ultrapure water; the Ca-ARS complexes were solubilised in 10% CPC buffer and the absorbance was read at 575 nm. The values are expressed as the relative mineralization compared with 7-days old (D0) cells.

Silencing of TRPM7 gene. For gene silencing of the TRPM7 channel, cells were transfected for 48 h with small interfering RNA (siRNA) targeting the TRPM7 mRNA sequence ATGGAATTTAAAGAAGTA (si-TRPM7) or with nontargeting negative control siRNA (si-Mock) as described previously [14].

Reverse-transcription polymerase chain reaction. Total RNA was isolated using the TriZol reagent (Invitrogen) according to manufacturer's recommendations. Two μg of total RNA were reversed transcribed using the QIAGEN RT kit (Qiagen, Mississauga, Ontario, Canada), followed by a 40 cycle PCR (Labnet Multigene II) at a 58°C annealing temperature using the QIAGEN PCR kit (Qiagen) with specific primers for GAPDH and TRPM7 [14], as well as for the osteoblast differentiation marker osteocalcin (OCN) (F: 5'-CAAGTCCCACACAGCAGCTT-3', R: 5'-

AAAGCCGAGCTGCCAGAGTT-3'). The PCR products were visualized with ethydium bromide staining following an electrophoresis on 2% agarose gel.

Statistical analysis. Statistical differences were evaluated by Two-tailed Student's *t*-test or ANOVA with Bonferroni post-test using GraphPad Prism 4. A level of $P<0.05$ was considered significant.

Results and discussion

Bone formation relies on the osteoblastic functions such as proliferation, migration, differentiation, secretion of matrix proteins [2]. Our previous results have indicated that TRPM7 channels are involved in the intracellular Ca/Mg homeostasis of osteoblastic cells as well as in basal and PDGF-induced cell proliferation and migration [11, 12]. Since studies have reported that insufficient dietary Mg intake is associated with reduced levels of serum alkaline phosphatase and osteocalcin, which suggest a decrease in osteoblastic differentiation [8, 9], we investigated the importance of Mg and TRPM7 channels in the osteoblastic differentiation of murine MC3T3 cells. These cells provide a well-known in vitro model for studying the differentiation process from preosteoblast to mature osteoblast [13] using well-established culture conditions [15]. As shown in Fig. 1A, differentiation treatments increased the activity of osteoblastic differentiation marker alkaline phosphatase (ALP) [15, 16], as a function of days in culture. As shown in Fig. 1B, differentiation treatments also increased by 2-fold the level of osteocalcin mRNA (1.03 ± 0.06 and 0.90 ± 0.12 for D0 and D28B respectively vs 2.04 ± 0.17 for D28T; student t test: $^{**}P<0.002$ compared with D28B from three independent experiments). To note, cellular proliferation stops at confluence (D0) and osteoblasts secrete significant quantities of extracellular matrix which will mineralized. In accordance, mineralization bone-like nodules were significantly increased in 28-days culture induced to differentiate compared with basal culture condition (Fig. 1C). Taken

together, these results confirm the efficiency of cell treatment to promote MC3T3 osteoblastic differentiation. Our previous results have indicated that TRPM7 channel is involved in the intracellular Ca and Mg homeostasis of osteoblastic cells [11]. Therefore, we tested the effect of differentiation treatments on the expression of TRPM7 channels, and we showed that the gene expression of TRPM7 is up-regulated by differentiation treatments (Fig. 2). The enhanced expression of TRPM7 was specific since the gene expression of TRPM6, a close homologue of TRPM7, was not modified (data not shown).

Since TRPM7 are Ca/Mg channels, we studied the influence of culture conditions with reduced extracellular Mg and Ca levels on the MC3T3 differentiation. Differentiation treatments were conducted for 28 days with culture media containing basal 1.36 mM Ca and 0.8 mM Mg (CTL), 0.34 mM Ca and 0.8 mM Mg (low Ca) or 1.36 mM Ca and 0.1 mM Mg (low Mg). As shown in Fig.3A, a significant reduction of ALP activity was measured under conditions of low extracellular Mg. Similar results was observed with low extracellular Ca concentrations. Furthermore, low extracellular Ca or Mg decreased the level of osteocalcin (OCN) gene expression determined by semi quantitative RT-PCR (Fig.3B), (0.52 ± 0.11 and 0.58 ± 0.04 for low Ca and Mg respectively vs 1.00 ± 0.08 for CTL condition; student t test: $^{**}P<0.005$ compared with control condition from three independent experiments). Our results show that culture conditions with low extracellular Ca or Mg levels reduced the mineralization (Fig. 3C). To note, cell viability was not compromised under culture of low Ca or Mg concentrations (data not shown). Therefore, our results

indicate a significant and specific reduction of MC3T3 differentiation and mineralization of extracellular matrix under culture conditions with low Ca or low Mg levels. It should be noted that differentiation and mineralization was not prevent under latter conditions but rather reduced globally by 40-60% since the levels of osteoblastic differentiation markers under differentiation culture conditions with low extracellular Ca or Mg were still higher compared to untreated cells under normal condition of Ca or Mg (data not shown).

To directly test whether TRPM7 is involved in osteoblast differentiation and mineralization, MC3T3 cells were transfected with small interfering RNAs (siRNA) targeting TRPM7 channels (si-TRPM7) or non targeting siRNAs (si-Mock) each three days of culture to maintain reduction of TRPM7 expression for 28 days. Analysis by semi quantitative RT-PCR confirmed the specific reduction of TRPM7 gene expression by siRNA treatments (Fig. 4A). Silencing TRPM7 gene expression in MC3T3 cells during 28 days of culture prevented the increase of ALP activity as shown in Fig. 4B. Furthermore silencing TRPM7 gene decreased the level of OCN (Fig. 4C) (1.04 ± 0.04 and 1.06 ± 0.14 for CTL and si-Mock respectively vs 0.67 ± 0.05 for si-TRPM7; student t-test: $^{*}P<0.02$ compared with si-Mock condition from three independent experiments) and reduced the mineralization (Fig 4D). These results suggest that adequate expression of TRPM7 is essential for murine osteoblast differentiation and mineralization of extracellular matrix.

Conclusion

Our current results indicate that extracellular Ca, Mg and TRPM7 are important for murine osteoblast differentiation. Reduction of osteoblastic differentiation under impaired Mg levels would lead to inadequate bone formation resulting in the development of osteoporosis.

Acknowledgements

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References

[1] S. Harada, G.A. Rodan, Control of osteoblast function and regulation of bone mass, Nature 423 (2003) 349-355.

[2] S.C. Manolagas, Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis, Endocr. Rev. 21 (2000) 115-137.

[3] E. Mackie, Osteoblasts: novel roles in orchestration of skeletal architecture, Int. J. Biochem. Cell Biol. 35 (2003) 1301-1305.

[4] R.K. Rude, H.E. Gruber, Magnesium deficiency and osteoporosis: animal and human observations, J. Nutr. Biochem. 15 (2004) 710-716.

[5] M.A. Kenney, H. McCoy, L. Williams, Effects of magnesium deficiency on strength, mass, and composition of rat femur, Calcif. Tissue Int. 54 (1994) 44-49.

[6] A.L. Boskey, C.M. Rimnac, M. Bansal, M. Federman, J. Lian, B.D. Boyan, Effect of short-term hypomagnesemia on the chemical and mechanical properties of rat bone, J. Orthop. Res. 10 (1992) 774-783.

[7] R.K. Rude, M.E. Kirchen, H.E. Gruber, M.H. Meyer, J.S. Luck, D.L. Crawford, Magnesium deficiency-induced osteoporosis in the rat: uncoupling of bone formation and bone resorption, Magnes. Res. 12 (1999) 257-267.

[8] T.O. Carpenter, S.J. Mackowiak, N. Troiano, C.M. Gundberg, Osteocalcin and its message: relationship to bone histology in magnesium-deprived rats, Am. J. Physiol. 263 (1992) E107-E114.

[9] A. Creedon, A. Flynn, K. Cashman, The effect of moderately and severely restricted dietary magnesium intakes on bone composition and bone metabolism in the rat, Br. J. Nutr. 82 (1999) 63-71.

[10] J.E. Aubin, Advances in the osteoblast lineage, Biochem. Cell Biol. 76 (1998) 899-910.

[11] E. Abed, R. Moreau, Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation, Cell. Prolif. 40 (2007) 849-865.

[12] E. Abed, R. Moreau, Importance of Melastatin-like Transient Receptor Potential 7 and Magnesium in the Stimulation of Osteoblast Proliferation and Migration by PDGF, Am. J. Physiol. Cell Physiol. (2009) (in press).

[13] W.J. Peterson, K.H. Tachiki, D.T. Yamaguchi, Serial passage of MC3T3-E1 cells down-regulates proliferation during osteogenesis in vitro, Cell. Prolif. 37 (2004) 325-336.

[14] E. Abed, D. Labelle, C. Martineau, A. Loghin, R. Moreau, Expression of transient receptor potential (TRP) channels in human and murine osteoblast-like cells, Mol. Membr. Biol. 26 (2009) 146-158.

[15] M. Yamauchi, T. Yamaguchi, H. Kaji, T. Sugimoto, K. Chihara, Involvement of calcium-sensing receptor in osteoblastic differentiation of mouse MC3T3-E1 cells, Am. J. Physiol. Endocrinol. Metab. 288 (2005) E608-E616.

[16] J. Jadlowiec, H. Koch, X. Zhang, P.G. Campbell, M. Seyedain, C. Sfeir, Phosphophoryn regulates the gene expression and differentiation of NIH3T3, MC3T3-E1, and human mesenchymal stem cells via the integrin/MAPK signaling pathway, J. Biol. Chem. 279 (2004) 53323-53330.

Legends to the figures

Fig. 1 Osteoblastic differentiation of MC3T3 cells. Cells were grown to confluence (day 0) or further incubated for 7, 14, 21 and 28 days in basal media (basal) or in differentiation medium (treated). **A)** Alkaline phosphatase (ALP) activity was measured as described in the materials and methods section. Significant differences compared with the activity in basal condition (*$p<0.05$, ***$p<0.0001$, Bonferoni). **B)** Total RNA was isolated from cells grown to confluence (D0) or further incubated for 28 days in basal (D28B) or differentiating condition (D28T). Representative results of the relative levels of osteocalcin (OCN) expression compared with the gene expression of GAPDH determined by semi quantitative RT-PCR are shown. **C)** Mineralization was assessed by staining mineralized nodules with alizarine red. Student t test: ***$p<0.0003$ compared with basal 28 days condition (D28B) from three independent experiments.

Fig. 2 Expression of TRPM7 channels along osteoblastic differentiation. Cells were grown to confluence (D0) or further incubated for 28 days in basal media (D28B) or in differentiation medium (D28T). Total RNA was isolated and relative levels of TRPM7 expression were determined by semi quantitative RT-PCR. Student t test: *$P<0.03$ compared with the basal 28-days condition from three independent experiments.

Fig. 3 Influence of reduced extracellular magnesium and calcium on the osteoblast differentiation. Cells were incubated for 28 days in differentiating culture media

containing 1.36 mM Ca and 0.8 mM Mg (CTL), 0.34 mM Ca and 0.8 mM Mg (low Ca) or 1.36 mM Ca and 0.1 mM Mg (low Mg). **A)** Alkaline phosphatase (ALP) activity was measured as described in the materials and methods section. Student t test: *P<0.04, **P<0.002 compared with the control condition from three independent experiments. **B)** Total RNA was isolated from cells cultured under previous conditions. Representative results of the relative levels of osteocalcin (OCN) expression compared with gene expression of GAPDH determined by semi quantitative RT-PCR are shown. **C)** Mineralization was assessed by staining mineralized nodules with alizarine red. Student t test: ***p<0.0002, **p<0.002 compared with control condition from three independent experiments.

Fig. 4 Effect of reducing TRPM7 expression on osteoblastic differentiation. Cells were treated with differentiation media and transfected with specific siRNAs against mouse TRPM7 (si-TRPM7) or with nontargeting control siRNA (si-Mock) twice a week for 28 days. A) The expression of human TRPM7 in MC3T3 cells was determined after 48 h by RT-PCR and normalized according to the expression of GAPDH for 3 independent experiments. Student t test: ***P<0.0002 compared with si-Mock condition from three independent experiments. B) Alkaline phosphatase (ALP) activity was measured as described previously. Student t test: *P<0.02, compared with si-Mock condition from three independent experiments. C) Total RNA was isolated from cells cultured under previous conditions, and relative levels of osteocalcin (OCN) expression were determined by semi quantitative RT-PCR. D) Mineralization was assessed by staining mineralized nodules cells with alizarine red.

Student t test: **p<0.0002, compared with si-Mock condition from three independent experiments.

Fig. 1 Osteoblastic differentiation of MC3T3 cells.

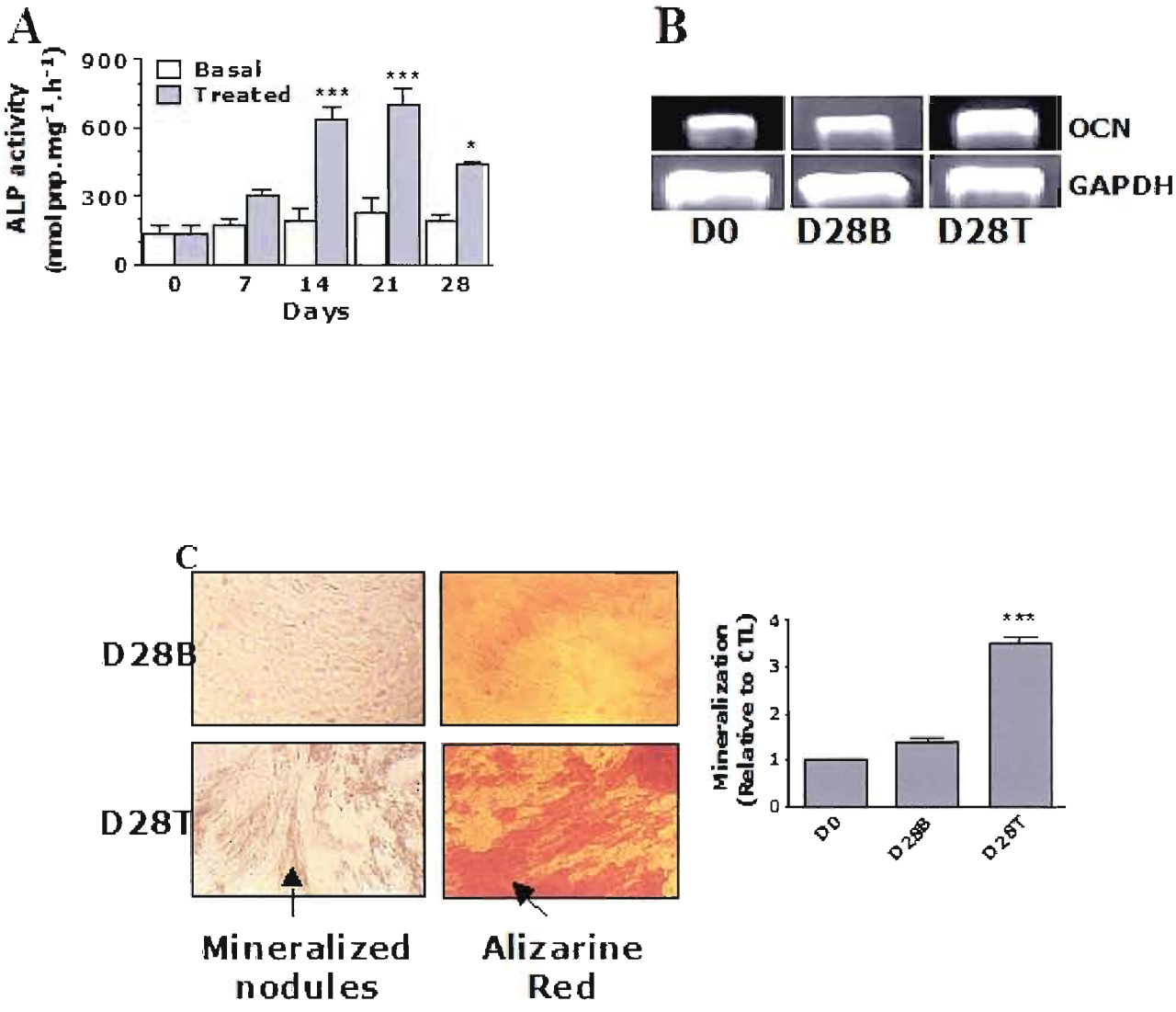


Fig. 2 Expression of TRPM7 channels along osteoblastic differentiation

Fig. 3 Influence of reduced extracellular magnesium and calcium on the osteoblast differentiation

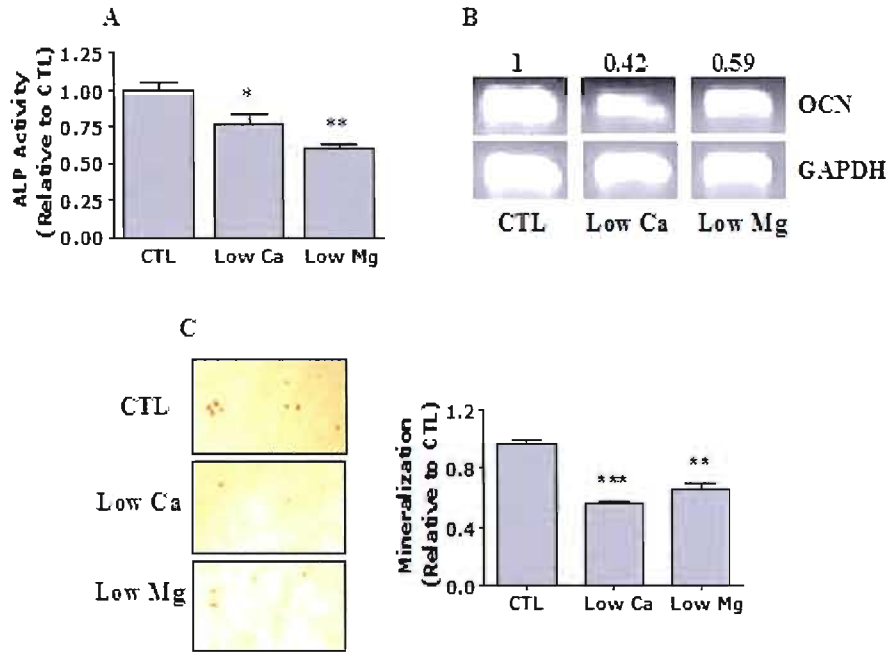
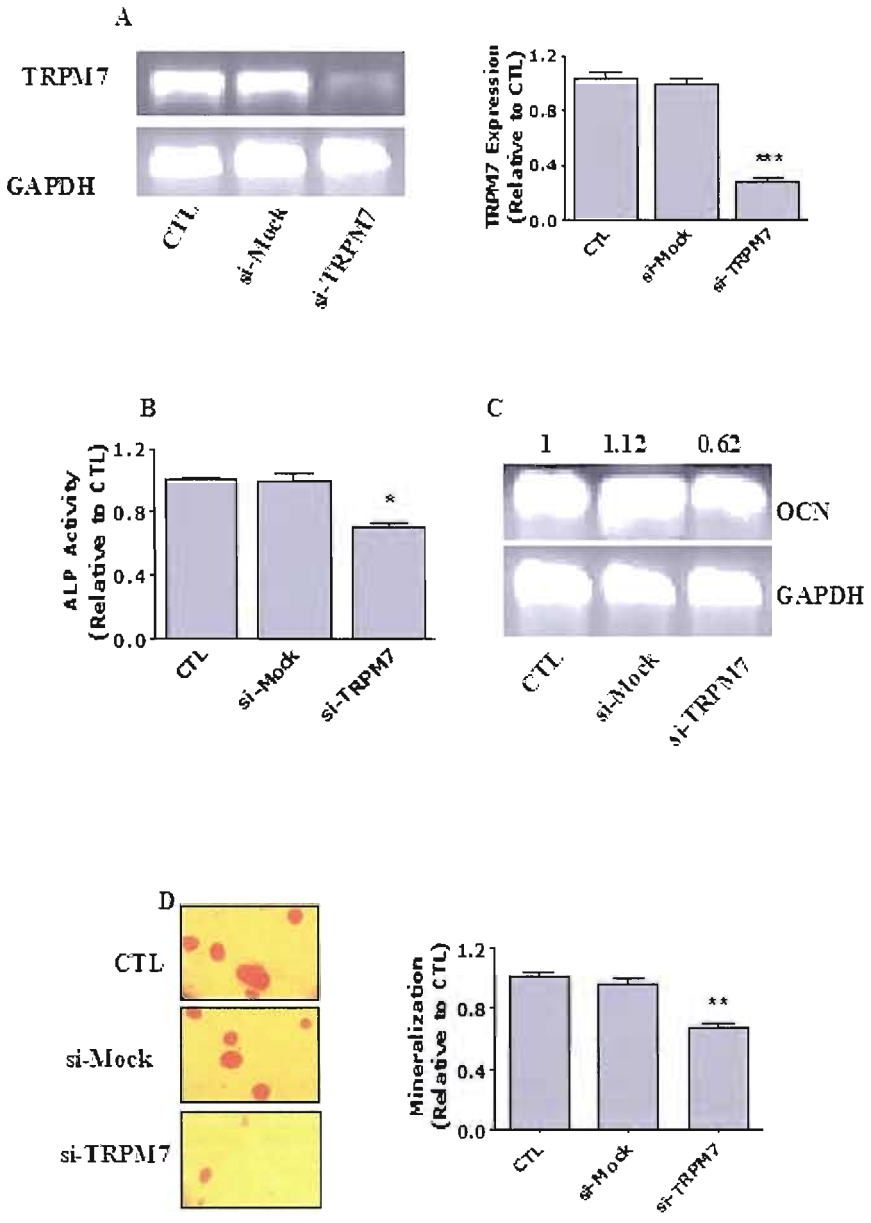


Fig. 4 Effect of reducing TRPM7 expression on osteoblastic differentiation.



CHAPITRE III

3.1. CONCLUSION

Le remodelage osseux est un processus biologique dont l'équilibre est primordial afin de préserver les propriétés biomécaniques du tissu osseux et d'assurer l'homéostasie minérale ainsi que de réparer les fractures. Dans bien des cas où l'équilibre est perdu, il y a apparition d'ostéoporose qui est caractérisée par une masse osseuse réduite due à une dégradation osseuse supérieure à sa formation. En raison de l'augmentation de la longévité de la population et de son incidence croissante avec l'âge, l'ostéoporose est ainsi devenue un véritable problème majeur de santé publique (Bonura 2009). L'étude de la régulation du remodelage osseux devient primordiale afin de mieux comprendre les mécanismes menant à la perte de la masse osseuse et à l'ostéoporose. Puisque les ostéoblastes sont les cellules impliquées dans la formation osseuse et qu'elles contrôlent aussi la différenciation et l'activation des ostéoclastes, ces cellules sont désormais considérées comme une cible importante dans la prévention et le traitement de l'ostéoporose. Ainsi la compréhension des mécanismes fondamentaux de signalisation cellulaire et de régulation des fonctions ostéoblastiques est d'une importance primordiale dans le développement de meilleures approches médicales pour la prévention et le traitement de l'ostéoporose. Les facteurs qui augmentent le risque de développer l'ostéoporose comprennent l'âge, le genre, les antécédents familiaux, l'ascendance européenne ou asiatique, une constitution morphologique mince ou petite, l'inactivité physique, le tabagisme, un régime pauvre en Ca et en vitamine D, la consommation d'alcool et la prise de certains médicaments (Fujiwara 2009).

Une formation osseuse adéquate repose sur les nombreuses fonctions des ostéoblastes telles la prolifération, la migration et la différenciation mais également

sur l'activité sécrétoire de ces cellules en vue de synthétiser et minéraliser la matrice osseuse. Le Ca^{2+} ainsi que le Mg^{2+} sont impliqués dans plusieurs processus biologiques entre autres la prolifération, la différenciation, ainsi que la mort cellulaire (apoptose). Toutefois, les mécanismes assurant l'homéostasie du Ca^{2+} et du Mg^{2+} intracellulaire ne sont pas encore parfaitement élucidés. Plusieurs études épidémiologiques ont montré une corrélation directe entre le déficit modéré en Mg^{2+} et la réduction de la masse osseuse. Ainsi une diète faible en Mg^{2+} a été identifiée comme un facteur prédisposant à une réduction graduelle de la masse osseuse et au développement de l'ostéoporose chez l'humain (Rude et Gruber 2004). De plus chez le rat et la souris, une diète déficiente en Mg^{2+} entraîne une augmentation du nombre d'ostéoclastes matures, une diminution du nombre d'ostéoblastes et une perte de la masse osseuse sans que la calcémie et les niveaux de vitamine D ne soient altérés (Rude R et al 2005).

L'homéostasie du Mg^{2+} ainsi que sa régulation au niveau des ostéoblastes ne sont pas encore parfaitement élucidées. Nous présentons dans cette étude des résultats soulignant l'importance du Mg^{2+}, sa régulation et l'implication des canaux «melastatin related transient receptor potentiel 7» (TRPM7) au niveau de la prolifération, de la migration et de la différenciation des ostéoblastes ainsi que leur capacité à synthétiser et minéraliser la matrice osseuse.

Nos travaux indiquent pour la première fois que les cellules ostéoblastiques expriment une grande variété de canaux de la superfamille des canaux TRP. Ces canaux cationiques sont à même d'intervenir dans plusieurs processus biologiques, pourtant peu d'informations sont disponibles sur leur fonctions dans les cellules osseuses et plus spécifiquement au niveau de l'homéostasie du Ca^{2+} et du Mg^{2+} dans les cellules ostéoblastiques.

Nos travaux ont démontré l'expression de divers TRPC par les ostéoblastes. Ces canaux sont principalement impliqués dans l'influx calcique. De façon générale, les canaux TRPC sont activés par le relargage des réserves de Ca^{2+} intracellulaires

(mode d'activation désigné «Ca^{2+} release-activated Ca^{2+} channel», CRACC ou «store-operated Ca^{2+} channel», SOCC), activés par un récepteur (processus nommé «receptor-operated Ca^{2+} channel», ROCC) et activés par des seconds messagers (régulation appelée «second messenger-operated Ca^{2+} channels», SMOCC). Nos résultats indiquent que les canaux TRPC1 sont impliqués dans l'influx calcique généré selon un mécanisme d'activation de type SOCC lors de l'ajout de Ca extracellulaire suite à une stimulation avec le PDGF. De plus une stratégie d'interférence à l'ARN ciblant TRPC1 prévient l'influx calcique généré par les SOCC ainsi que la prolifération des cellules ostéoblastiques induite par le PDGF. Pourtant la réduction de l'ARNm de TRPC3 n'avait aucun effet sur l'influx calcique de type SOCC et sur la prolifération cellulaire. Nous croyons donc que l'influx de Ca^{2+} de type SOCC est nécessaire à la prolifération des ostéoblastes induite par le PDGF et est dépendant de TRPC1. Ces résultats suggèrent l'implication des canaux TRPC1 dans le processus précoce de la formation osseuse qui implique une expansion des cellules ostéoblastiques qui seront sollicitées pour la synthèse de la matrice osseuse. Il est à noter qu'en plus d'exprimer divers canaux TRP, plusieurs variants d'épissage des canaux TRPC ont été identifiés chez les mammifères ce qui augmente la diversité de la réponse calcique. Ainsi, la diversité de la réponse calcique des ostéoblastes en regard des différents facteurs de régulation du métabolisme osseux pourrait prendre naissance dans l'expression de multiples isoformes et de variants d'épissage de TRPC, et également dans les mécanismes d'activation de ces canaux.

Nos résultats montrent une réduction de la prolifération cellulaire des ostéoblastes dans les conditions de culture faible en Mg^{2+} suggérant que ce cation est essentiel pour une prolifération optimale. Ces résultats concordent très bien avec la littérature, entre autre avec l'hypothèse émise en 1975 par Harry Rubin indiquant que le Mg^{2+} joue un rôle primordial et contrôle le processus de prolifération cellulaire. Diverses études ont aussi rapporté une diminution de la prolifération chez les cellules

endothéliales (Bernardini D et al 2005) et les cellules mammaires de souris (HC11) (Sgambato A et al 1999) en fonction de la réduction du Mg^{2+} extracellulaire. À cet égard, nos mesures de synthèse d'ADN ont indiqué qu'une réduction de Mg dans le milieu de culture diminue la synthèse de l'ADN, et par le fait même la prolifération des ostéoblastes sans affecter leur phénotype, suggérant un rôle important du Mg^{2+} dans la régulation de cycle cellulaire. Harry Rubin a rapporté que le Mg^{2+} contrôle les différentes phases du cycle cellulaire (G0/G1, S, G2/M). Il a montré que la réduction du Mg^{2+} extracellulaire entraîne un arrêt de croissance et a observé une augmentation du pourcentage des cellules en phase G0/G1, une diminution en phase S ainsi qu'une légère augmentation de cellules en phase G2/M. D'un point de vue moléculaire, plusieurs équipes ont montré que les cellules incubées dans un milieu de culture faible en Mg expriment davantage les gènes *p27* (Fererice I el al 2008), *p21* (Bernardini, D 2005) et *p35*, (les protéines p27, p21 et p35 sont connues comme des inhibiteurs du cycle cellulaire) (Figure 1). Parallèlement, la réduction du Mg^{2+} dans le milieu de culture entraîne une diminution du facteur de transcription F2F (Prolactin receptor), ce dernier initie la transcription d'ARNm nécessaire à la phase S du cycle cellulaire. (Toyoda M et al 2000; Petersen PH et al 2002).

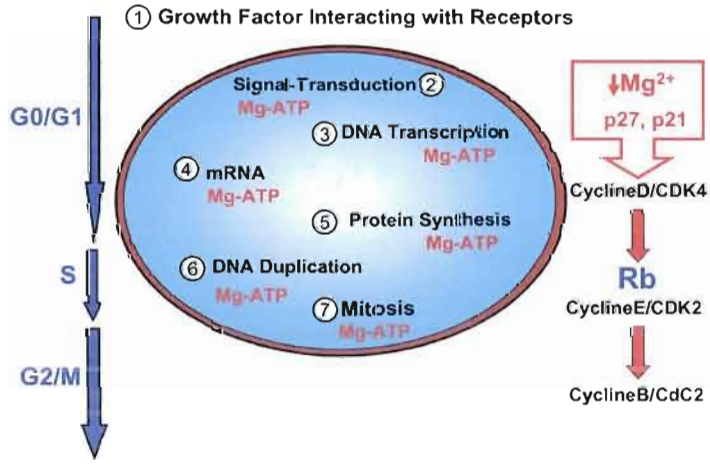


Figure 1 : Magnésium et prolifération cellulaire.
Tiré de (Federica I. Wolf et Valentina Trapani 2008)

En vue de son rôle important dans la prolifération cellulaire, la compréhension de la régulation du Mg^{2+} intracellulaire est primordiale. Plusieurs canaux et transporteurs membranaires assurent l'homéostasie du Mg^{2+} cytosolique. Les canaux TRPM6 et TRPM7 de la grande famille des TRP, ont été identifiés comme étant des canaux transportant le Mg^{2+}. Récemment, Goytain et Quamme (2005) ont identifié plusieurs transporteurs membranaires du Mg^{2+} (SLC41A1, SLC41A2, MagT1 et ACDP2).

Nos travaux ont indiqué que les ostéoblastes expriment les canaux TRPM6 et TRPM7 et que les canaux cationiques TRPM7 favorisent l'influx calcique et du Mg^{2+} afin de maintenir l'homéostasie des ces cations et d'assurer une prolifération adéquate. De plus, nos résultats montrent que la réduction de l'expression de TRPM7 réduit la prolifération cellulaire des ostéoblastes. Nos résultats sont en accord avec la littérature.

Plusieurs études ont observé une corrélation entre l'expression des canaux TRPM7 et la prolifération au niveau des cellules du rétinoblastome (Hanano *et al.* 2004) et des cellules musculaires lisses vasculaires (He *et al.* 2005). Une réduction de l'expression de TRPM7 dans les cellules HEK293 entraîne un arrêt de la prolifération et la mort cellulaire (Nadler MJS et al 2001). Il a été montré que la prolifération de ces cellules pouvait également être retrouvée en augmentant le niveau du Mg^{2+} extracellulaire Nadler MJS et al 2001). Par contre nos résultats indiquent que l'ajout du Mg^{2+} dans le milieu de culture ne renverse pas la réduction de la prolifération cellulaire des ostéoblastes. Nous suggérons que les canaux TRPM6 et les autres transporteurs membranaires (SLC41A1, SLC41A2, MagT1 et ACDP2), même s'ils sont exprimés dans les cellules ostéoblastiques, sont incapables de compenser la diminution de la prolifération cellulaire. Effectivement, Sahni et al (2007) ont indiqué que les cellules DT40 déficientes en TRPM7 sont incapables de compenser la réduction de la prolifération cellulaire lors de l'ajout du Mg^{2+} dans le milieu de culture même s'ils expriment le transporteur membranaire du Mg^{2+} (SLC41A2).

Il est aussi intéressant de noter que nos résultats indiquent que l'expression génique de TRPM7 des ostéoblastes est augmentée en condition de culture où le Mg^{2+} et le Ca^{2+} sont réduits. De plus, la réduction du Mg^{2+} extracellulaire entraîne une activation des canaux TRPM7 se traduisant par des influx calciques et du Mg^{2+}. Cette augmentation d'expression ainsi que l'activation des canaux TRPM7 permettent aux cellules de compenser le déficit en Ca^{2+} et Mg^{2+}, et de maintenir une concentration intracellulaire adéquate des ces cations en favorisant l'influx. In vivo, au niveau rénal l'expression des SLC41A1, MagT1 et ACDP2 est principalement augmentée chez les souris en condition d'hypomagnésie (Goytain et Quamme 2005). De même, dans les cellules rénales l'expression des MagT1 et ACDP2 est augmentée dans les conditions de culture où le Mg^{2+} est réduit, suggérant un mécanisme de compensation afin de maintenir et de conserver l'homéostasie du Mg^{2+} de l'organisme en augmentant l'absorption du Mg (Goytain et Quamme 2005). De ce fait, les canaux TRPM7 peuvent être ajoutés à la liste des transporteurs de Mg^{2+} dont l'expression est modulée par le Mg^{2+}.

Durant le processus de remodelage osseux, les ostéoblastes vont migrer vers les lacunes de résorption, proliférer pour entamer par la suite le processus de formation osseuse. Ces processus de migration et de prolifération sont influencés par divers facteurs de croissance présents dans le microenvironnement osseux. Ainsi, nous avons évalué l'importance des canaux TRPM7 dans la prolifération et la migration des ostéoblastes induite par le PDGF. Ce dernier est un facteur connu pour stimuler la prolifération et la migration de plusieurs types cellulaires, entre autre des ostéoblastes. Tout d'abord, nos résultats montrent que les ostéoblastes humains MG-63 ont une migration basale et cette migration est amplifiée par la stimulation par le PDGF. Pour la première fois, nos résultats indiquent que la réduction du Mg^{2+} extracellulaire inhibe la migration basale et induite par le PDGF, ainsi que la prolifération des ostéoblastes MG-63 induite par le PDGF. Les cellules en migration vont projeter des prolongements cytoplasmiques (ce qui reflète un changement de

morphologie cellulaire et la réorganisation du cytosquelette) et modifier le niveau d'adhésion cellulaire. Nos essais de trypsinisation ont démontré que la réduction du Mg du milieu de culture entraîne une diminution de l'adhésion cellulaire basale et stimulée par le PDGF. De plus, nous avons observé que la réduction du Mg^{2+} (0.1mM) du milieu de culture change la morphologie des cellules (moins de prolongements cytoplasmiques) et change la réorganisation du cytosquelette, déterminée par la visualisation des filaments d'actine en microscopie confocale. Toutefois, la morphologie cellulaire ainsi que la réorganisation du cytosquelette restent inchangées dans les conditions de culture faible en Ca^{2+} (0.1mM) indiquant l'importance du Mg^{2+} dans l'adhésion et la migration des MG-63.

Considérant que nos résultats indiquent que l'effet du PDGF sur la prolifération et la migration dépend du Mg^{2+} extracellulaire, nous avons formulé l'hypothèse que le PDGF favorise un influx du Mg^{2+} pour stimuler la prolifération ainsi que la migration des ostéoblastes humains. Effectivement, nos résultats montrent que le PDGF favorise l'influx du Mg^{2+}. De plus, la stimulation des MG-63 par le PDGF augmente l'expression génique de TRPM7 et cette régulation de l'expression est spécifique aux canaux TRPM7 puisque l'expression de son homologue, le canal TRPM6, demeure inchangée.

De plus, la réduction de l'expression génique de TRPM7 par l'interférence à l'ARN réduit l'influx du Mg^{2+} et abolit la prolifération ainsi que l'adhésion et la migration basale et induite par le PDGF des ostéoblastes humains MG-63. Ainsi, nos résultats démontrent l'implication de TRPM7 dans l'effet du PDGF.

Ainsi nos résultats montrent que la réduction de l'expression de TRPM7 réduit l'adhésion et la migration des ostéoblastes MG-63. Il est à noter que les canaux TRPM7 représentent un modèle de ''*chanzyme*'' et ainsi possèdent une extrémité C-terminale avec un domaine kinase qui intervient par un mécanisme d'autophosphorylation dans l'activité du canal TRPM7 et aussi phosphoryle

l'annexine et la chaîne lourde de la myosine IIA (19). Il est possible que l'effet observé sur l'adhésion et la migration dans notre modèle dépend de l'activité du canal et/ou de son domaine kinase. Pour mieux répondre, il faudra recourir à la mutagenèse dirigée. (Clark et al 2006) ont montré, chez les cellules leucémiques basophiles (RBL) de rats, que le domaine kinase du canal TRPM7 phosphoryle la chaîne lourde de la myosine II et participe à la stabilisation des filaments d'actine, et qu'une surexpression des canaux TRPM7 entraîne un changement structural et favorise l'adhésion cellulaire. Par contre, les travaux de Li-Ting Su et al (2006) ont suggéré que les canaux TRPM7 contrôlent l'adhésion cellulaire en favorisant un influx calcique, ce dernier stimulant la m-calpain, une protéase dépendante du Ca^{2+} capable de réguler les protéines retrouvées au sein des complexes d'adhérence. De plus, ils montrent que la réduction de l'expression des canaux TRPM7 favorise plutôt l'adhésion cellulaire ainsi que la mobilité cellulaire chez les cellules de rein d'embryon humain (HEK). Ainsi, nos résultats concordent avec les travaux de Clark et al mais s'opposent à ceux de Li-Ting Su et al.

Il est bon de noter que les travaux de (Sugden EA et Nihei T 1969) suggèrent que le Ca^{2+} ainsi que le Mg^{2+} peuvent se lier au chaîne myosine, et que le Mg^{2+} montre un effet direct et influence l'activité enzymatique. D'un autre côté, le Mg^{2+} contrôle la régulation du niveau du Ca^{2+} intracellulaire, et de ce fait le Mg^{2+} a été considéré comme un modulateur principal des activités liées au Ca^{2+}. Un changement au niveau du Mg^{2+} cellulaire peut entraîner une modification du Ca^{2+}, et par la suite cet ion peut moduler l'activité de m-calpain et la chaîne lourde de la myosine II et ainsi agir sur l'adhésion et la migration cellulaire, suggérant que l'effet observé est probablement lié à la réduction du Mg^{2+} intracellulaire. Globalement, les études indiquent l'importance du Mg^{2+} dans le processus de l'adhésion et la migration cellulaire.

Pour entamer le processus du remodelage osseux, les ostéoblastes vont se différencier en ostéoblastes matures, synthétiser et secréter la matrice organique et

assurer sa minéralisation en modulant la déposition d'hydroxyapatite (Manolagas 2000). Les cellules ostéoprogénitrices MC3T3 constituent un excellent modèle *in vitro* de différenciation des précurseurs ostéoblastiques en ostéoblastes matures avec l'ajout dans le milieu de culture de l'acide ascorbique et du glycérol-2-phosphate (Minguell *et al* 2001). L'analyse des marqueurs de différenciation des ostéoblastes (ostéocalcine et phosphatase alcaline) et les mesures de minéralisation (rouge alizarine) ont indiqué pour la première fois qu'une réduction du Ca^{2+} ou du Mg^{2+} du milieu de culture diminue la capacité des cellules ostéoprogénitrices MC3T3 à se différencier en ostéoblastes matures et à minéraliser la matrice osseuse. De plus, les cellules MC3T3 en voie de différenciation exprimaient davantage le gène TRPM7. Par ailleurs, une stratégie d'interférence à l'ARN ciblant TRPM7 a réduit la capacité des MC3T3 à se différencier et par la suite à minéraliser la matrice osseuse. En conclusion, nos résultats montrent que la différenciation des cellules ostéoprogénitrices MC3T3 en ostéoblastes matures est favorisée par la présence des cations Ca/Mg extracellulaires et des canaux TRPM7.

Certaines maladies telles l'hypertension, les maladies nerveuses, l'athérosclérose sont associées au carence en Mg^{2+} (Laires *et al.* 2004). De très nombreux médicaments sont aussi responsables de pertes importantes de Mg^{2+}. Un excès de Ca^{2+} ou de potassium sous forme de suppléments pourrait entraîner une carence en Mg^{2+} (Rosanoff A 2007). L'organisme a tendance à maintenir une concentration sérique en Mg^{2+} stable. Ainsi, le taux de Mg^{2+} dans le sang ne reflète pas l'état des réserves accumulées dans les tissus. Un déficit tissulaire en Mg^{2+} peut être observé malgré une magnésémie normale. Plusieurs travaux ont confirmé l'absence de relation directe entre la magnésémie et les concentrations intra-tissulaires de Mg^{2+} (Touyz RM 2004). Plus de la moitié du Mg^{2+} de l'organisme se trouve dans l'os, où il participe à la formation de la matrice osseuse et contribue à la fixation du Ca^{2+} dans la matrice osseuse ce qui en fait une réserve importante pour l'organisme. En effet, une partie du Mg^{2+} est mobilisable en cas de déficit. Il faut noter qu'une relation entre le taux de Mg^{2+} osseux et l'ostéoporose a été observée ; le contenu en

Mg^{2+} dans les os, et particulièrement dans l'os trabéculaire, est réduit de 10 à 18% chez des patients souffrant d'ostéoporose (Bunker 1994).

Pour mieux comprendre la corrélation entre le Mg^{2+} et l'ostéoporose, divers scénario peuvent être envisagés. Lors de la formation osseuse les ostéoblastes secrètent des vésicules matricielles qui contrôlent le dépôt minéral (Ca^{2+}, phosphates, Mg^{2+}, zinc et autres) dans des microdomaines de la matrice. Ce dépôt dépend de la concentration de chaque ion dans la circulation. Lors d'une hypomagnésie, il se peut que les ostéoblastes déposent dans la matrice ossseuse une quantité de Mg^{2+} inférieure à la normal. De plus lors du remodelage osseux, les ostéoclastes dégradent la matrice osseuse en libérant des produits de dégradation, entre autre le Ca^{2+} et le Mg^{2+}. Cette phase est suivie d'une phase de formation pendant laquelle les ostéoblastes vont migrer vers le site de la résorption, proliférer, se différencier, synthétiser et minéraliser la matrice osseuse. Dans une condition normale les ostéoblastes vont être exposés à une concentration normale en Mg^{2+}. Dans le cas d'une hypomagnésie ou le contenu en Mg^{2+} sérique est réduit, la libération du Mg^{2+} dans le microdomaine sera inférieure à la concentration normal en Mg^{2+}. Ainsi, les ostéoblastes seront exposés à des niveaux de Mg^{2+} inférieurs à la normale, ce qui pourra affecter les fonctions des ostéoblastes et par la suite la formation osseuse. Ce phénomène va s'amplifier dans le temps et aboutir à une réduction de la masse osseuse et par la suite à l'ostéoporose.

3.2. PERSPECTIVES

Nos résultats révèlent que le canal TRPM7 joue un rôle dans les fonctions des ostéoblastes et dans le processus du remodelage osseux. De plus, le TRPM7 a la propriété d'un canal et présente une activité enzymatique capable de moduler l'activité du canal et d'autres substrats. Pour mieux comprendre si l'effet observé sur les fonctions des ostéoblastes est dû à l'activité du canal seul, du domaine kinase ou à l'effet combiné du canal et du domaine kinase, nos prochaines étapes pour améliorer cette étude seraient d'utiliser la mutagenèse dirigée qui consiste à introduire des mutations dans des régions bien déterminées du domaine kinase afin de le rendre inactif et par la suite mieux élucider l'importance du canal TRPM7 et de son domaine kinase sur les fonctions des ostéoblastes.

De plus il serait intéressant de vérifier l'effet du Mg^{2+} et l'implication des canaux TRPM7 au niveau de la prolifération, la migration et la différenciation de cultures primaires d'ostéoblastes afin de mieux apprécier ses rôles *in vivo.* Il faut dire que le TRPM7 semble essentiel pour le développement embryonnaire, puisque la délétion du gène TRPM7 chez la souris est létale (Nadler MJ 2001). Les cellules progénitrices extraites de la moelle osseuse des os de souris représentent un modèle qui peut être utilisé afin de déterminer le rôle du TRPM7 dans la différenciation car ces cellules sont capables de se différencier en ostéoblastes dans des conditions de culture appropriées. Ces cellules pourraient être utilisées afin de déterminer l'importance du Mg^{2+} et des canaux TRPM7 au niveau de la différenciation ostéoblastique afin d'évaluer les impacts et les usages cliniques potentiels.

Cette étude souligne l'importance du Mg^{2+} et des canaux TRPM7 au niveau des fonctions des cellules ostéoblastiques et nos résultats se veulent en accord avec la diminution de la formation osseuse et le développement de l'ostéoporose associés à une diète déficiente en Mg^{2+}. Enfin, nous croyons que cette étude apportera les fondements pour des traitements ciblés de l'ostéoporose.

3.3. RÉFÉRENCES

Ambudkar IS, Ong HL, Liu X, Bandyopadhyay BC, Cheng KT. (2007) Trpc1: the link between functionally distinct store-operated calcium channels. Cell Calcium. Aug;42(2):213-23.

Abdel-Latif AA. (1986) Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. Pharmacol Rev, 38: 227-272.

Anast CS, Forte LF. (1983) Parathyroid function and magnesium depletion in the rat. Endocrinology. Jul;113(1):184-9.

Aubin JE, Bonnelye E. (2000) Osteoprotegerin and its ligand: A new paradigm for regulation of osteoclastogenesis and bone resorption. Medscape Womens Health. Mar; 5(2):5.

Aubin JE. Regulation of osteoblast formation and function. (2001) Rev Endocr Metab Disord. Jan; 2(1):81-94. Review.

Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL, Jordt SE, Julius D. (2007) The menthol receptor TRPM8 is the principal detector of environmental cold. Nature. Jul 12;448(7150):204-8.

Bergman RJ, Gazit D, Kahn AJ, Gruber H, McDougall S, Hahn TJ. (1996) Age-related changes in osteogenic stem cells in mice. J. Bone Miner. Res. 11, 568-577.

Bernardini, D., Nasulewicz, A., Mazur, A. and Maier, J. A. (2005) Magnesium and microvascular endothelial cells: a role in inflammation and angiogenesis. Front. Biosci. 10, 1177–1182.

Billiau A, Edy VG, Heremans H, Van Damme J, Desmyter J. (1977) Human interferon: mass production in a newly established cell line, MG-63, Antimicrob. Agents Chemother. 12(1): 11-15.

Biologie humaine : anatomie et physiologie par Elaine N.Marieb, René Lachaine. Sixième édition page 114.

Bonewald LF, Dallas SL. (1994) Role of active and latent transforming growth factor beta in bone formation. J Cell Biochem. Jul;55(3):350-7.

Bonura F. (2009) Prevention, screening, and management of osteoporosis: an overview of the current strategies. Postgrad Med. Jul; 121(4):5-17.

Bootman MD, Lipp P, Berridge MJ. (2001) The organisation and functions of local Ca(2+) signals. J. Cell Sci.; 114: 2213-2222.

Boulay G, Brown DM, Qin N, Jiang M, Dietrich A, Zhu MX, Chen Z, Birnbaumer M, Mikoshiba K, Birnbaumer L. (1999) Modulation of Ca(2+) entry by polypeptides of the inositol 1,4, 5- trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): evidence for roles of TRP and IP3R in store depletion-activated Ca(2+) entry. Proc. Natl. Acad. Sci. U. S. A; 14955-14960.

Brauchi S, Orta G, Mascayano C, Salazar M, Raddatz N, Urbina H, Rosenmann E, Gonzalez-Nilo F, Latorre R. (2007) Dissection of the components for PIP2 activation

and thermosensation in TRP channels. Proc Natl Acad Sci U S A. Jun 12;104(24):10246-51.

Calvino B. (2003) Heat hyperalgesia during inflammation: involvement of TRPV1. Med Sci (Paris). Jun-Jul;19(6-7):649-51.

Catterall WA. (2000) Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol;16:521-55. Review.

Chen TL. (2004) Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. Bone 35, 83-95

Christakos S. Vitamin D gene regulation. (1996) Principles of bone biology. New York: Academic Press: 435-46.

Christakos S, Dhawan P, Liu Y, Peng X, Porta A. (2003) New insights into the mechanisms of vitamin D action. J Cell Biochem. Mar 1;88(4):695-705. Review.

Claesson-Welsh L. (1996) Mechanism of action of platelet-derived growth factor. Int J Biochem Cell Biol. Apr;28(4):373-85.

Clapham, D.E. (2007) Calcium signaling. Cell, 131:1047-1058.

Clapham DE, Runnels LW, Strubing C. (2001) The TRP ion channel family. Nat. Rev. Neurosci. 2, 387-396.

Clapham, D. E., Runnels, L. W. and Strübing, C. (2001). The TRP Ion Channel Family. *Nature Reviews Neuroscience* 2, 387-396.

Clark K, Langeslag M, van Leeuwen B, Ran L, Ryazanov AG, Figdor CG, Moolenaar WH, Jalink K, van Leeuwen FN. (2006) TRPM7, a novel regulator of actomyosin contractility and cell adhesion. *EMBO J* 25: 290-301.

Cohen-Solal M, de Vernejoul MC. (2003) Bone remodeling. Therapie. Sep-Oct;58(5):391-3. Review.

Demion M, Bois P, Launay P, Guinamard R. (2007) TRPM4, a Ca2+-activated nonselective cation channel in mouse sino-atrial node cells. Cardiovasc Res. Feb 1;73(3):531-8.

Devescovi V, Leonardi E, Ciapetti G, Cenni E. (2008) Growth factors in bone repair. Chir Organi Mov. Dec;92(3):161-8. Review.

Essig M, Amiel C. (1977) Désordres de la magnésémie. In : Offenstadt G, Brunette MG, eds. Désordres acido-basiques et hydro-électrolytiques. Paris : Arnette Blackwell ; p 379-406.

Ertel EA, Campbell KP, Harpold MM. (2000) Nomenclature of voltage-gated calcium channels. *Neuron* ; 25 : 533-5.

Fauconnier J, Lanner JT, Sultan A, Zhang SJ, Katz A, Bruton JD, Westerblad H. (2007) Insulin potentiates TRPC3-mediated cation currents in normal but not in insulin-resistant mouse cardiomyocytes. Cardiovasc Res. Jan 15;73(2):376-85.

Federica I. WOLF and Valentina TRAPANI. (2008) Cell (patho)physiology of magnesium. Clinical Science (2008) 114, 27–35.

Franz-Odendaal TA, Hall BK, Witten PE. (2005) Buried alive: How osteoblasts become osteocytes.Dev Dyn. Oct 28.

Fredriksson L, Li H, Eriksson U. (2004) The PDGF family: four gene products form five dimeric isoforms. Cytokine Growth Factor Rev. Aug;15(4):197-204. Review.

Fujiwara S. (2009) Fracture risk assessment tool. Clin Calcium. Sep;16(9):1487-91.

Gehron Robey P. (1989) The biochemistry of bone. Endocrinol Metab Clin North Am. Dec;18(4):858-902. Review.

Goytain A, Quamme GA. (2005a) Functional characterization of ACDP2 (ancient conserved domain protein), a divalent metal transporter. Physiol. Genomics 22, 382-389.

Goytain A, Quamme GA. (2005b) Functional characterization of human SLC41A1, a Mg^{2+} transporter with similarity to prokaryotic MgtE Mg^{2+} transporters. Physiol. Genomics 21, 337-342.

Goytain A, Quamme GA. (2005c) Functional characterization of the human solute carrier, SLC41A2. Biochem. Biophys. Res. Commun. 330, 701-705.

Goytain A, Quamme GA. (2005d) Identification and characterization of a novel mammalian Mg^{2+} transporter with channel-like properties. BMC. Genomics 6, 48.

G T Rijkers and A W Griffioen. (1993) Changes in free cytoplasmic magnesium following activation of human lymphocytes. Biochem J. 1993 January 15; 289(Pt 2): 373–377.

Hanano T, Hara Y, Shi J, Morita H, Umebayashi C, Mori E, Sumimoto H, Ito Y, Mori Y, Inoue R. (2004) Involvement of TRPM7 in cell growth as a spontaneously activated Ca^{2+} entry pathway in human retinoblastoma cells. *J Pharmacol Sci* 95: 403-419.

Heldin CH, Hammacher A, Nistér M, Westermark B. (1988) Structural and functional aspects of platelet-derived growth factor. Br J Cancer. Jun;57(6):591-3. Review.

H Rubin and T Koide. (1976) Mutual potentiation by magnesium and calcium of growth in animal cells. : Proc Natl Acad Sci U S A. Jan;73(1):168-72.

Hubbard KB, Hepler JR. (2006) Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins.Cell Signal. Feb;18(2):135-50. Review.

Jayaraman T, Marks AR. (1997) T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. Mol Cell Biol. Jun;17(6):3005-12.

Jordt SE, Ehrlich BE. (2007) TRP channels in disease.Subcell Biochem: 45:253-71.

Kanatani M, Sugimoto T, Kanzawa M, Yano S, Chihara K. (1999) High extracellular calcium inhibits osteoclast-like cell formation by directly acting on the calcium-sensing receptor existing in osteoclast precursor cells. Biochem Biophys Res Commun. Jul 22;261(1):144-8

Kraft R, Harteneck C. (2005) The mammalian melastatin-related transient receptor potential cation channels: an overview. *Pflugers Arch*; 451: 204-11.

Kiselyov K, Kim JY, Zeng W, Muallem S. (2005) Protein-protein interaction and functionTRPC channels. Pflugers Arch. Oct;451(1):116-24. Review.

Kotev-Emeth S, Savion N, Pri-chen S, Pitaru S. (2000) Effect of maturation on the osteogenic response of cultured stromal bone marrow cells to basic fibroblast growth factor. Bone 27, 777-783.

Laires MJ, Monteiro CP, Bicho M. (2004) Role of cellular magnesium in health and human disease. *Front Biosci* 9: 262-276.

Large WA. (2002) Receptor-operated Ca2(+)-permeable nonselective cation channels in vascular smooth muscle: a physiologic perspective Cardiovasc Electrophysiol. May;13(5):493-501.

Large WA, Saleh SN, Albert AP. (2009) Role of phosphoinositol 4,5-bisphosphate and diacylglycerol in regulating native TRPC channel proteins in vascular smooth muscle. Cell Calcium. Jun;45(6):574-82.

Larissa Lipskaia and Anne-Marie Lompré. (2004) Alteration in temporal kinetics of Ca^2 signaling and control of growth and proliferation. Biol Cell. Feb;96(1):55-68.

Li X, Udagawa N, Itoh K, Suda K, Murase Y, Nishihara T, Suda T, Takahashi N (2002), p38 MAPK-mediated signais are required for inducing osteoclast differentiation but not for osteoclast function. *Endoerinology* 143, 31053113.

Liang CT, Barnes J, Seedor JG, Quartuccio HA, Bolander M, Jeffrey JJ, Rodan GA. (1992) Impaired bone activity in aged rats: alterations at the cellular and molecular levels. Bone 13, 435-441.

Lopez-Ilasaca M. (1998) Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. Biochem. Pharmacol.; 56: 269-277.

Mackie E. (2003) Osteoblasts novel roles in orchestration of skeletal architecture. *Int JBiochem Cell Biol* 35: 1301-1305.

Malathi K, Kohyama S, Ho M, Soghoian D, Li X, Silane M, Berenstein A, Jayaraman T. (2003) Inositol 1,4,5-trisphosphate receptor (type 1) phosphorylation and modulation by Cdc2. J. Cell Biochem. 90: 1186-1196.

Norbert Weiss, Michel De Waard (2006). Médecine/sciences ; 22 : 396-404

Mehrotra M, Krane SM, Walters K, Pilbeam C. (2004) Differential regulation of platelet-derived growth factor stimulated migration and proliferation in osteoblastic cells. *J Cell Biochem* 93: 741-752,

Monteilh-Zoller MK, Hermosura MC, Nadler MJ, Scharenberg AM, Penner R, Fleig A. (2003) TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. J. Gen. Physiol. 121, 49-60.

Marie PJ. Osteoblasts and bone formation. In : Zaidi M, ed. (1999) Advances in organ biology :molecular and cellular biology of bone. Stamford, CT (USA): JAI Press; 5B: 401-27.

Muraki K, Iwata Y, Katanosaka Y. (2003) TRPV2 is a component of osmotically sensitive cation channels in murine aortic myocytes. *Circ Res*; 93 : 829-38.

Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurosaki T, Kinet JP, Penner R, Scharenberg AM, Fleig A. (2001) LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. *Nature* 411: 590-595.

Nargeot, J., and Charnet, P. (1994). Diversité moléculaire des canaux calciques: du gène à la fonction. *médecine/sciences* 10, 1293-1308

Nilius B, Mahieu F, Prenen J, Janssens A, Owsianik G, Vennekens R, Voets T. (2006) The Ca^{2+}-activated cation channel TRPM4 is regulated by phosphatidylinositol 4,5-biphosphate. EMBO J 25: 467-478.

O'Neil RG, Heller S. (2005) The mechanosensitive nature of TRPV channels. Pflugers Arch ; 451 : 193-203.

Owsianik G, Talavera K, Voets T, Nilius B. (2006) Permeation and selectivity of TRP channels. Annu Rev Physiol.68:685-717.

Pierre Marie 2001 médecine/sciences; 17 : 1252-9.

Pedersen SF, Owsianik G, Nilius B. (2005) TRP channels: an overview. Cell Calcium 38: 233-252.

Putney JW, Jr. (1986) A model for receptor-regulated calcium entry. Cell Calcium; 7: 1-12.

Quarto R, Thomas D, Liang CT. (1995) Bone progenitor cell deficits and the age-associated decline in bone repair capacity. Calcif. Tissue Int. 56, 123-129.

R.K. Rude, H.E. Gruber, L.Y. Wei, A. Frausto and B.G. Mills. (2003) Magnesium deficiency: effect on bone and mineral metabolism in the mouse, Calcif Tissue Int. 72), pp. 32–41.

Roholl PJ, Blauw E, Zurcher C, Dormans JA, Theuns HM. (1994). Evidence for a diminished maturation of preosteoblasts into osteoblasts during aging in rats: an ultrastructural analysis. J. Bone Miner. Res. 9, 355-366.

Romani, A. and Maguire, M. E. (2002) Hormonal regulation of Mg2+ transport and homeostasis in eukaryotic cells. Biometals 15, 271–283.

Rosanoff A. (2005) Magnesium and hypertension. Clin Calcium. Feb;15(2):255-60. Review.

Rubin, H. (1975) Central role for magnesium in coordinate control of metabolism and growth in animal cells. Proc. Natl. Acad. Sci. U.S.A. 72, 3551–3555.

Rubin, H. & D.F. Bowen-Pope. (1979) Coordinate control of Balb/c3T3 cell survival and multiplication by serum or calcium pyrophosphate complexes. J.Cell. Physiol. 98, 81-94.

Rubin, H. & T. Koide. (1976) Mutual potentiation by magnesium and calcium of growth in animal cells. Proc. Natl. Acad. Sci. USA, 73, 168-172.

Rude R, Gruber H, Norton H, Wei L, Frausto, J. (2005) Kilburn Dietary magnesium reduction to 25% of nutrient requirement disrupts bone and mineral metabolism in the rat. Bone 37: 211-219.

Rude RK, Gruber HE. (2004) Magnesium deficiency and osteoporosis: animal and human observations. *J Nutr Biochem* 15: 710-716.

Sahni J, Nelson B, Scharenberg AM. (2007) SLC41A2 encodes a plasma-membrane Mg^{2+} transporter. *Biochem J* 401: 505-513.

Sanui, H. & H. Rubin (1977) Correlated effect of external magnesium on cation content and DNA synthesis in cultured chicken embryo fibroblasts. J. Cell. Physiol. 92, 23-32.

Scharenberg AM. (2005) TRPM2 and TRPM7: channel/enzyme fusions to generate novel intracellular sensors. *Pflugers Arch*; 451 : 220-7.

S.C. Manolagas. (2000) Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis, Endocr. Rev. 21: 115-137.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang M-S, Luethy R et al. (2005). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell ;89:309-19.

Singh BB, Lockwich TP, Bandyopadhyay BC, Liu X, Bollimuntha S, Brazer SC, Combs C, Das S, Leenders AG, Sheng ZH, Knepper MA, Ambudkar SV, Ambudkar IS. (2004) VAMP2-dependent exocytosis regulates plasma membrane insertion of TRPC3 channels and contributes to agonist-stimulated Ca2+ influx. Mol Cell. Aug 27;15(4):635-46.

Sorkin AM, Dee KC, Knothe Tate ML. (2004) "Culture shock" from the bone cell's perspective: emulating physiological conditions for mechanobiological investigations, *Am.1 Physiol Cel! Physiol287:* C1527-C1536.

Smyth, J.T., Dehaven, W.I., Jones, B.F., Mercer, J.C., Trebak, M., Vazquez, G., and Putney, J.W. Jr. (2006) Emerging perspectives in store-operated Ca2+ entry: roles of Orai, Stim and TRP. Biochim Biophys Acta, 1763: 1147-1160.

Strewler GJ. (2001) Local and systemic control of the osteoblast. J Clin Invest; 107: 271-2.

Sugden EA, Nihei T. (1969) The effects of calcium and magnesium ions on the adenosine triphosphatase and inosine triphosphatase activities of myosin A. Biochem J. Aug;113(5):821-7.

Su LT, Agapito MA, Li M, Simonson WT, Huttenlocher A, Habas R, Yue L, Runnels LW. (2006) TRPM7 regulates cell adhesion by controlling the calcium-dependent protease calpain. *J Biol Chem* 281: 11260-11270.

Tatibana M, Ishijima S, Kita K, Ishizuka T, Suzuki N. (1989) Early mitogenic stimulation of metabolic flux through phosphoribosyl pyrophosphate into nucleotides in Swiss 3T3 cells and requirement of external magnesium for the response. Adv Enzyme Regul ;28:147-66.

Touyz RM. (2004) Magnesium in clinical medicine. Front Biosci. May 1;9:1278-93.

Vassort G, Alvarez J. (2009) Transient receptor potential: a large family of new channels of which several are involved in cardiac arrhythmia. Can J Physiol Pharmacol. Feb;87(2):100-7.

Wenstrup RJ, Witte OP, Florer JB. (1996) Abnormal differentiation in MC3T3-E 1 preosteoblasts expressing a dominant-negative type 1 collagen mutation, *Connect. Tissue Res.* 35: 249-257.

Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C. (1995) TRPC1, a human homolog of a Drosophila store-operated channel. Proc. Natl. Acad. Sci. U. S. A; 92: 9652-9656.

Xu H, Zhao H, Tian W, Yoshida K, Roullet JB, Cohen DM. (2003) Regulation of a transient receptor potential (TRP) channel by tyrosine phosphorylation. SRC family kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its response to hypotonic stress. J Biol Chem. Mar 28;278(13):11520-7.

Yamakage M, Namiki A. (2002) Calcium channels--basic aspects of their structure, function and gene encoding; anesthetic action on the channels--a review. Can J AnaesthFeb;49(2):151-64. Review

Yao F, Schaffer PA. (1995) An activity specified by the osteosarcoma line U20S can substitute functionnally for ICPO, a major regulatory protein of Herpes simplex virus type 1, *J Virology* 69(10): 6249-6258.

Zhao W, Byrne MH, Wang Y, Krane SM. (2000) Osteocyte and osteoblast apoptosis and excessive bone deposition accompany failure of collagenase cleavage of collagen. J Clin Invest ; 106 : 941-9.

Zhu MX. Multiple roles of calmodulin and other Ca2+-binding proteins in the functional regulation of TRP channels. *Pflugers Arch* 2005 ; 451 : 105-15.

APPENDICE A

CHARACTERIZATION OF OXIDIZED LOW DENSITY LIPOPROTEINS-INDUCED HORMESIS-LIKE EFFECTS IN OSTEOBLASTIC CELLS

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Running head: Hormesis-like effect induced by oxLDL in osteoblasts

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ABSTRACT

Epidemiological studies indicate that patients suffering from atherosclerosis are predisposed to develop osteoporosis. Atherogenic determinants such as oxidized low density lipoprotein (oxLDL) particles have been shown both to stimulate the proliferation and promote apoptosis of bone-forming osteoblasts. Given such opposite responses, we characterized the oxLDL-induced hormesis-like effects in osteoblasts. Biphasic MTT reductive activity responses were induced by oxLDL where low concentrations (10-50 microg/ml) increased and high concentrations (from 150 microg/ml) reduced the MTT activity. Cell proliferation stimulation by oxLDL partially accounted for the increased MTT activity. No alteration of mitochondria mass was noticed whereas low concentrations of oxLDL induced mitochondria hyperpolarization and increased the cellular levels of reactive oxygen species (ROS). The oxLDL-induced MTT activity was not related to intracellular ROS levels. OxLDL increased NAD(P)H-associated cellular fluorescence and flavoenzyme inhibitor DPI reduced basal and oxLDL-induced MTT activity, suggesting an enhancement of NAD(P)H-dependent cellular reduction potential. Low concentrations of oxLDL reduced cellular thiol content and increased metallothionein expression suggesting the induction of compensatory mechanisms for the maintenance of cell redox state. These concentrations of oxLDL reduced osteoblast alkaline phosphatase activity and cell migration. Our results indicate that oxLDL particles cause hormesis-like response with the stimulation of both proliferation and

cellular NAD(P)H-dependent reduction potential by low concentrations, while high concentrations lead to reduction of MTT activity associated with the cell death. Given the effects of low concentrations of oxLDL on osteoblast functions, oxLDL may contribute to the impairment of bone remodeling equilibrium.

Keywords: **osteoblasts; atherosclerosis; oxLDL; oxysterol; hormesis**

INTRODUCTION

Elevated levels of serum low-density lipoprotein (LDL) particles are considered as the most important atherogenic risk factor. LDL particles are thought to become atherogenic after undergoing oxidative modifications and key roles of oxidized LDL (various oxidized species collectively designated oxLDL) in atherosclerosis have been largely reviewed by Steinberg (46). Oxidized LDL and oxysterols have been shown to increase or decrease proliferation, and trigger apoptosis process depending on the cell types related to vasculature alterations, nature of oxLDL and oxysterols, and on the concentrations used (52); thus supporting numerous deleterious effects that sustain the development of atherosclerosis. Also, a number of clinical studies suggest an association between cardiovascular diseases and the development of osteoporosis, independently of age and hormonal deficiency (2, 3, 48-50). Positive relationships with LDL particles and LDL-associated apoB both in men and women have been reported for osteoporosis (1, 37, 41). Lipid accumulation has been observed in osteoporotic and aging bone (36, 42, 47). The presence of oxidized lipids was revealed in the bone marrow of hyperlipidemic mice (47). Furthermore Maggio et al (33) have reported a marked decrease in plasma antioxidants in aged osteoporotic women. Therefore, these studies suggest the existence of one or several contributory factors for the parallel development of atherosclerosis and osteoporosis.

The bone is a dynamic tissue that is continuously being remodelled following two opposite and coordinated processes. Under normal conditions, specialized cells called

osteoclasts transiently break down old bone (resorption process) at multiple sites as other cells known as osteoblasts are replacing it with new tissue (bone formation). Following differentiation from mesenchymal stem cells, osteoblastic cells assure bone formation and mineralization through the secretion of bone matrix components (type I collagen and noncollagenous proteins), and also play a central role in the regulation of bone resorption by providing essential factors such as macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-kappaB ligand (RANKL) for the differentiation of osteoclasts (32). In this context, alteration of osteoblastic proliferation, differentiation, secretory functions or apoptosis rate are though to compromise the maintenance of bone remodelling equilibrium. Parhami et al. (40) reported a reduction of bone mineralization in mice fed with an atherogenic high-fat diet, with a decreased expression of osteoblastic marker osteocalcin by marrow cells suggesting an inhibition of osteoblastic differentiation. Accordingly oxLDL particles have been reported to promote *in vitro* cell proliferation and to inhibit the differentiation of murine osteoprogenitor cell line MC3T3-E1 and of bone marrow osteoblastic precursor cells (38, 39). Liu et al. (29) showed that low concentrations of oxysterol cholestane-3b,5a,6b-triol increased cell viability and that high concentrations inhibited osteoblastic differentiation and promoted the apoptosis of primary rat bone marrow stromal cells. Klein et al (20) reported an inhibition of the osteoblastic phenotype marker alkaline phosphatase activity and cell death by oxLDL in human osteoblastic SaOS cells. We have reported that high concentrations of oxLDL cause cell death through the apoptosis of human osteoblastic MG-63 cells

(6). Altogether, the studies have reported paradoxical stimulation and loss of osteoblastic viability by oxLDL which have highlighted oxLDL as a contributory factor for the parallel development of atherosclerosis and osteoporosis.

Given that the effects of oxLDL appear to be dependent on the concentrations, we speculated that the effects of oxLDL on cell viability may not correspond to typical monophasic dose-response but rather be associated with biphasic responses related to hormesis. Determination of dose-response effects is general procedure in toxicology for the risk evaluation and the establishment of exposure guidelines in view of monophasic responses. The MTT assay has been widely used for this end and has also permitted to reveal in some cases biphasic hormesis responses (13, 14, 43, 51). Hormesis has been defined as a dose-response relationship in which a stimulatory response occurs at low doses, and an inhibitory response takes place at high doses, resulting in a U- or inverted U-shaped dose response (10). Biphasic relationships have been described for various end point functions such as growth (metabolism, proliferation, survival, longevity) and deleterious effects (disease, cancer, etc). Hormesis is considered as an evolutionarily conserved process and the mechanisms underlying hormesis remain an enigma. The induction of biphasic hormesis-like relationships have been described for mild heat stress, radiation and by exposure to environmental toxic agents such as heavy metals (11). In some cases, hormesis has been considered as an adaptive or conditioning response that increases the resistance of the cell or organism from moderate to severe levels of stress. Such observations of

biphasic dose-response relationships have changed the general conception of the risk evaluation since the stimulation effect of low concentrations may result in the loss of regulation and equilibrium of cell functions, especially in view of bone remodeling

Given that opposite effects by oxLDL such as stimulation of cell viability and promotion of cell death have been reported in osteoblastic cells, we have characterized the hormesis-like effects induced by oxLDL in osteoblastic cells and focused on the effects of low concentrations of oxLDL on osteoblastic functions.

MATERIALS AND METHODS

Cell culture

Human osteoblastic MG-63, SaOS, U2 OS, and murine osteoprogenitor MC3T3 cells (ATCC, Rockville, MD, USA) were grown in a 1:1 mixture of phenol-free DMEM/Ham's F12 medium (DMEM/F12; Sigma, Oakville, Ontario, Canada) for MG-63, in McCoy's medium (Hyclone, Logan, UT, USA) for SaOS and U2 OS cells, and in alpha-MEM medium (Sigma, Oakville, Ontario, Canada) for MC3T3 cells. All media were supplemented with 10% fetal bovine serum (FBS; Cansera, Etobicoke, Ontario, Canada), L-glutamine (Invitrogen, Burlington, Ontario, Canada) and penicillin/streptomycin (Invitrogen, Burlington, Ontario, Canada). Cells were cultured in 5% CO_2 at 37 °C and were harvested weekly with Trypsin-EDTA solution (Invitrogen, Burlington, Ontario, Canada). Bone marrow stromal cells (BMSC) and primary osteoblasts (mOB) from C57BL/6 mice were isolated as described previously (12, 23). Briefly, mice were euthanased according to institution procedure for the use of experimental animals and the protocole was approved by the Institutional Animal Care and Use Committee of Université du Québec à Montréal. The femur and tibia were removed under aseptic conditions. Bones were broken in half and centrifuged for the collection of BMSC. Following a short spin, the cell pellets were re-suspended, seeded in 100 mm dishes (Sarstedt, Montréal, Québec, Canada), and allowed to adhere for two days in alpha-MEM medium (osteoblastic differentiation medium) supplemented with 15% FBS. The cells remaining in suspensions were washed out

and adherent cells were cultured for one to two weeks. For mOB, bone fragments were subjected to three consecutive digestions with collagenase A (Sigma) and digested-fragments were plated with alpha-MEM medium in 100mm dishes (Sarstedt) until cells outgrowth and reach confluence.

Isolation and modification of lipoproteins

Lipoprotein particles were isolated from human plasma obtained from Bioreclamation (Hicksville, NY, USA). Prior to the isolation, the plasma was adjusted to 0.01% ethylenediamine tetraacetate (EDTA), 0.02% sodium azide and 10 μM phenylmethylsulfonylfluoride (PMSF). Human LDL (d = 1.025-1.063 g/ml) were prepared as described by Brissette *et al.* (5). Lipoprotein particles contained no detectable amount of apoE as assessed by immunoblotting.

LDL preparations were dialysed against Tris-buffered saline (TBS) to remove EDTA before oxidation. Oxidized LDL particles were prepared as described by Lougheed and Steinbrecher (31). LDL particles (200 μg of protein/ml in TBS) were incubated with 5 μM $CuSO_4$ for 20h at 37°C. Oxidation was stopped by the addition of EDTA (final concentration of 100 μM) and butylated hydroxytoluene (40 μM final) and the oxLDL particles were concentrated to 15-20 mg/ml using Centriplus-100 ultrafiltration devices (Amicon, Oakville, Ont.). Oxidized LDL typically resulted in a 2.8-fold increase in the electrophoretic mobility relative to native LDL on 0.5 % agarose/barbital gels.

MTT reduction assay

For measurement of cell proliferation or viability, cells were seeded in 96-well plates (Sarstedt). After 5 days of culture in media containing 10% FBS, the cells were further incubated in DMEM/F12 without FBS in the absence or the presence of native LDL, oxidized LDL or oxysterols 7-ketocholesterol and 7β-hydroxycholesterol (Sigma, Oakville, Ontario, Canada). Two hours prior to the end of treatments, the media was replaced with DMEM/F12 containing 0.5 mg/ml of MTT (Sigma, Oakville, Ontario, Canada). Cellular reduction of the tetrazolium ring of MTT resulted in the formation of a dark purple water insoluble deposit, the formazan crystals. At the end of the incubation, media was aspired and formazan crystals were dissolved in DMSO. Absorbance was measured at 575 nm with a spectrophotometer and data was expressed as relative MTT activity corresponding to the ratio of absorbance of lipoprotein-treated cells vs control cells incubated with DMEM/F12 alone. In certain experiments, the cells were pretreated with chloroquine or diphenyleneiodonium (DPI) 1h prior to the addition of MTT or 24h with N-acetylcysteine (NAC) or l-buthionine-(S,R)-sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase, prior to treatment with oxLDL. Chloroquine diffuses into acidic compartments and becomes protonated, thereby destroying the acidic environment and inactivating the acid-dependent lysosomal enzymes. DPI phenylates and inhibits a variety of flavoenzymes, such as the mitochondrial NADH dehydrogenase (complexe I) and the NADPH oxidase.

Flow cytometry and confocal microscopy analysis

For cell division analysis, CarboxyFluorescein Succinimidyl Ester (CFSE, Invitrogen) was used. This cell permeable dye is de-esterified by intracellular enzymes creating a charged molecule trapped inside the cells. Upon division, daughter cells get half of the fluorescent marker and therefore reduction of fluorescence may be used to monitor cell division. CFSE (5 mM stock solution in DMSO) was added (final concentration of 2 μM) to the cells for 10 min at 37 °C. Labeling was stopped by the addition of 10% FBS for 15 min. CFSE labeled cells were cultured in vitro under different conditions. Cells were therefore trypsinized and analysed by flow cytometry with logarithmic detection of green fluorescence (CFSE). For cell counts, internal calibrator microspheres were added immediately prior to flow cytometric analysis. Using the cytofluorometer forward scatter and side scatter parameters, the interference of apoptotic cells and debris was excluded. Cell size was determined by the Forward side scatter function (SSC). Data were acquired in a FACScan flow cytometer (Becton Dickinson) using the Cell Quest software.

For the determination of mitochondria mass, the cells were incubated with 200 nM MitoTracker Green FM (In vitrogen) in DMEM/F12 for 30 minutes at 37°C, washed twice, and then analysed by a FACScan flow cytometer. This dye accumulates in the mitochondrial regardless of the membrane potential which allows the quantification of the amount of mitochondria. The mitochondrial membrane potential of intact cells was measured by flow cytometry with the lipophilic cationic probe 5,5′,6,6′-

tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Invitrogen). According to the mitochondrial potential, monomeric forms of JC-1 emit green fluorescence upon depolarization whereas their aggregation leads to red fluorescent with the hyperpolarization of mitochondria. Hence, the red/green fluorescent ratio is a way to measure the polarization, or potential energy of the mitochondria. Following treatments, the cells were trypsinized and the cell pellets were resuspended in 500 μl of PBS, incubated with 10 μM JC-1 for 20 min at 37°C. The cells were subsequently washed once with cold PBS, suspended in a total volume of 350 μl and analyzed by flow cytometer. The production of intracellular ROS was measured with the hydrogen peroxide-sensitive fluorescent dye carboxy-methyl dichlorofluorescein diacetate (CM-H2DCF-DA) (Invitrogen). This probe is non-fluorescent until cleavage by intracellular esterases, and its oxidation by intracellular hydrogen peroxide increases the fluorescence. Cells were incubated in culture media with CM-H2DCF-DA for 1h at a final concentration of 10 μM. Thereafter, cells were incubated at 37°C for various periods of time with oxLDL, washed with PBS, harvested and analyzed immediately by flow cytometry. For cellular autofluorescence measurements, treated cells were harvested and analyzed immediately by flow cytometry (λexc at 488 nm and λem at 530 and 570 nm).

For lysosomal staining, MG-63 cells were vitally stained with an acridine orange (AO, Sigma) solution at 5 μg/ml in complete medium for 30 minutes at 37°C. AO is a lysosomotropic weak base and a metachromatic fluorochrome showing red

fluorescence at high and green fluorescence at low concentrations. The intensities of red and green AO fluorescence were then examined with a laser scanning confocal (Bio-Rad) microscope (Nikon TE300) using a Plan-Apochromatic 60X oil N.A. 1.4 objective lens. For NAD(P)H-associated cellular fluorescence, the analysis was performed with CCD camera (λexc at 360 nm and λem at 460). For the measurements of cellular thiol content, cells were incubated in the culture medium or in the presence of oxLDL for 24 h. Thereafter, the cells were loaded with CMFDA for 30 min and the fluorescence was examined with a laser scanning confocal microscope, and analyzed with ImageJ software.

Metallothionein expression

Total RNA from cells was extracted using TriZol (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) reactions were carried out with Omniscript RT kit (Qiagen, Mississauga, Ontario, Canada) using hexamers. PCR amplifications were conducted with *Taq* PCR core kit (Qiagen) using specific primer sets for human metallothionein 1 and 2 (sense, 5'-TGGACCCCAACTGCTCCTGC-3'; antisense, 5'-GCCCTGGGCACACTTGGCAC-3') and for human GAPDH (sense, 5'-ACCACAGTCCATGCCATCAC-3'; antisense, 5'-TCCACCACCCTGTTGCTGTA-3'). Each primer was designed in distinct exons to ensure specific transcript amplification. Briefly, amplifications were carried out for 40 cycles according to

incubation of 1 min at 94 °C, 30 s at 58 °C and 1 min at 72 °C. Amplification products were resolved in 2% agarose gel and revealed by ethidium bromide staining.

Cell migration

In order to investigate the effects of oxLDL on MG-63 cell migration, a wound scratch assay was performed. Briefly, the cells were grown to confluent monolayer on 35mm diameter dishes (Sarstedt). The monolayers were wounded by scratching the surface as uniformly as possible with a pipette tip. This initial wounding and the movement of the cells in the scratched area was photographically monitored using the Axiovert Zeiss 200 microscope with a 10× (NA0.25) objective linked to a Coolsnap Es CCD camera for 24 hr. This time interval has been chosen because it is shorter than MG-63 doubling time in these conditions. Four different fields from each sample were considered for quantitative estimation of the number of cells that have migrated to the wounded area using imageJ software. The values are expressed as the relative cell migration compared to control condition in the culture medium.

Alkaline phosphatase activity

Measurement of alkaline phosphatase activity was performed by colorimetric assay of enzyme activity as described previously (35). Cell monolayers were washed three times with PBS buffer (0.1 g/l $CaCl_2$, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 0.1 g/l $MgCl_2.6H_2O$, 8 g/l NaCl, 1.44 g/l Na_2HPO_4, pH 7.4) and then scraped into assay buffer (100 mM glycine, 1mM $MgCl_2$, 1mM $ZnCl_2$, 1% Triton X-100, pH 10.5).

Assay were performed in 96-well plates with 75 µl of lysate mixed with 75 µl of the freshly prepared colorimetric substrate *para*-nitrophenyl phosphate (12 mM; Sigma, Oakville, Ontario, Canada) solubilized in the assay buffer. The enzymatic reaction was conducted for 1 h at 37 °C and was stopped by adding 100 µl of NaOH 0.2N. The optical density of the yellow product *para*-nitrophenol was determined spectrophotometrically at 410 nm. Alkaline phosphatase activity was expressed as *para*-nitrophenol produced in nmol/1h/mg of protein.

Cellular protein quantification

Cellular protein contents were quantified by MicroBCA protein assay (Pierce, Rockford, IL, USA) using BSA as standard.

Statistical analysis

Statistical differences were analysed by an ANOVA or Student t test using the GraphPad Prism3. A level of $P<0.05$ was considered as significant.

RESULTS

Effects of oxidized LDL on the MTT activity of MG-63 cells.

The levels of circulating oxLDL have been mentioned as a predictive and sensitive marker of coronary artery diseases. Holvoet et al. (17-19) have reported circulating concentrations of oxLDL ranging from 6-14 μg/ml for the patients showing no risk equivalent for coronary artery disease to 13-75 μg/ml for the patients with prevalence of coronary artery disease. In order to investigate both stimulation and inhibition responses corresponding to hormesis and reveal the effects of oxLDL at concentrations physiologically significant, we evaluated the effects of oxLDL by monitoring the MTT activity of osteoblastic cells exposed to concentrations ranging from 10 to 250 μg/ml. The MTT activity was determined in various models of human osteoblastic cells (MG-63, SaOS and U2 OS), murine osteoblastic MC3T3 cells, primary cultures of murine bone marrow stromal cells (BMSC) and osteoblasts from long bones (mOB) incubated with increasing concentrations of oxLDL. As shown in the Fig. 1A, oxLDL increased or decreased the MTT activity by osteoblastic cells after 48h of incubation according to the concentrations used (ANOVA, $P<0.0001$). At low concentrations, oxLDL increased the MTT activity of all osteoblastic models (Dunnett's $P<0.001$ from 10 μg/ml), whereas higher concentrations led to a reduction of the activity (Dunnett's $P<0.001$ from 150μg/ml for MG-63 and primary cultures of murine osteoblasts, and from 200μg/ml for the other cells). We previously reported that high concentrations of oxLDL could cause a decrease in MTT activity which

correlated to cell death by apoptosis, corroborated by Annexin V staining, the loss of lysosome integrity and DNA fragmentation (6). In contrast, native LDL increased the MTT activity of MG-63 cells, BMSC and mOB after 48h of incubation in a dose-dependent manner (ANOVA, $P<0.0001$). Native LDL particles did not alter the MTT activity of SaOS, U2 OS and MC3T3 cells. As shown in Fig. 1B, oxLDL (20μg/ml) increased the MTT activity (ANOVA, $P<0.001$), leading to a significant augmentation at 9h to 48 h (Dunnett's, $P<0.01$) whereas the MTT activity declined thereafter and was not significantly different from controls at 72h. A similar time-dependent decline of MTT activity was observed with MC3T3 cells (Fig. 1B). As oxLDL induced comparable hormesis-like biphasic responses in all the osteoblastic models analysed, subsequent experiments were performed with MG-63 which was the most responsive cell line.

Effects of oxysterols on MG-63 cells

We further evaluated the effects of 7β-hydroxycholesterol and 7-ketocholesterol, two oxysterols, detected in atherosclerotic lesions (7), formed by the copper-catalyzed oxidation of LDL on the MTT activity by MG-63 cells. As shown in Fig. 2A, low concentrations of 7β-hydroxycholesterol (from 5 μM) increased the MTT activity after 48 h (Dunnett's, $P<0.05$) whereas higher concentrations (above 30μM) led to a reduction of the activity (Dunnett's, $P<0.001$). On the other hand, increasing concentrations of 7-ketocholesterol reduced the MTT activity in a dose dependent manner (ANOVA, $P<0.001$) from 20μM (Dunnett's, $P<0.001$) (Fig. 2A). The

increase in MTT activity by 7β-hydroxycholesterol was shown to be time dependent (Dunnett's $P<0.01$) and as observed for oxLDL, was absent at 72 h (Fig. 2B). Lysophosphatidylcholine, another main component of oxLDL, did not alter the MTT activity of MG-63 cells (data not shown).

Effects of oxidized LDL on cell division of MG-63 cells

Given that an increased MTT activity is usually regarded as representative of cell proliferation, the effect of nLDL and oxLDL on division was further determined by estimation of total cell counts and measurement of the decrease in CFSE fluorescence, associated with cytokinesis. As shown in Fig. 3A and B, the number of cells and the relative cell division, determined by the decrease in CFSE fluorescence occurred in the presence of 200μg/ml of nLDL which confirms the stimulation of cell proliferation by nLDL as suggested by the increased MTT activity. In addition, low concentrations of oxLDL (10 and 20μg/ml) also increased significantly the cell number and relative cell division, As expected, concentrations of oxLDL from 50 μg/ml significantly reduced the cell number (Dunnett's, $P<0.01$) and reduced cell division evaluated by CFSE fluorescence when compared to low cell proliferation rate in control condition with the culture medium alone (Fig. 3A and B). It should be noted that cell number with 50 μg/ml of oxLDL was not different compared to basal (Basal) condition prior to the 48h-period of incubation which indicates the absence of cell proliferation. Furthermore when the relative MTT activity was normalized according to the relative cellular protein content (Fig. 3C, ANOVA $P<0.0001$) or cell

number (Fig. 3D), ratio above 1 was revealed for cells treated with 20 and 50μg/ml of oxLDL which argues for a significant discrepancy between the MTT activity and cell proliferation at these concentrations. Nevertheless, for concentrations of oxLDL above 100μg/ml ratio of MTT activity on cellular protein or cell number was approximately 1 confirming osteoblastic cell death as we have previously reported (6). Therefore, our results indicate that part of the increased MTT activity induced by low concentrations of oxLDL corresponds to cell proliferation and that high concentrations of oxLDL induced cell death which are characteristic of the oxLDL-induced hormesis-like effect. As a discrepancy between the MTT activity and cell proliferation at low concentrations of oxidized LDL was observed, the nature of the increased MTT activity promoted by 10, 20 and 50 μg/ml of oxLDL was studied.

MTT activity and lysosomal function

As oxLDL particles are known to disturb lysosomal function (27) and as MTT activity has been associated with endosome/lysosome (30), the correspondence between oxLDL-induced increased MTT activity and lysosome activity was evaluated. As shown in the Fig. 4A, incubation of MG-63 cells with low concentrations of oxLDL for 48 h led to an increase in cell autofluorescence which suggests the presence of undegradable lipofuscin/ceroid-like materials. The mitochondrial respiratory chain inhibitor sodium azide had no effect on basal or oxLDL-induced cellular autofluorescence suggesting that the latter fluorescence may be associated with an accumulation of lipofuscin/ceroid material (data not shown).

We hypothesized that a compensatory increase of cell endosome/lysosome activity could account for the increased MTT activity. As shown in the Fig. 4B, no increase in the cellular content of acidic compartments was revealed in cells incubated for 24h with low concentrations of oxLDL following the staining of acidic organelles with acridine orange. Moreover the inhibition of lysosomal function by the addition of chloroquine prior to MTT activity assays did not alter the MTT activity of the cells either in the absence or presence of low concentrations of oxLDL (Fig. 4B).

Effects of oxidized LDL on mitochondrial mass and cell size

Cellular hypertrophy has been reported in human umbilical vein endothelial cells exposed to oxLDL (44) and could account for the discrepancy between the MTT activity due to increased cell volume and organelle mass without an increase in cell number. Therefore, we evaluated the effect of oxLDL on mitochondria mass and cell size. Results in Fig. 5A show that low concentrations of oxLDL do not increase the mitochondrial mass of MG-63 cells (ANOVA, P=0.1680). Similar results were obtained with 7β-hydroxycholesterol and therefore increased mitochondrial mass excluding that it could account for the increased MTT activity. Moreover oxLDL and 7β-hydroxycholesterol did not affect the cell size of MG-63 cells (data not shown).

Effects of oxidized LDL on the mitochondrial membrane potential and the production of ROS

As the increase in the MTT activity of MG-63 cells by oxLDL may results from an imbalance in mitochondrial activity, the mitochondria membrane potential was

measured using JC-1. As shown in Fig. 5B, low concentrations of oxLDL increased the mitochondrial membrane potential in MG-63 cells. For comparison, incubation of MG-63 cells with the known mitochondria uncoupler FCCP was associated with mitochondria depolarization as revealed by reduced ratio of red/green fluorescence (Fig. 5B). As the hyperpolarization of mitochondria has been associated with the production of ROS, the levels of ROS in MG-63 cells incubated with low concentrations of oxLDL were further measured. As shown in Fig. 5C, the experimental concentrations of oxLDL used induced the production of ROS in MG-63 cells. Similarly to oxLDL, low concentrations of 7β-hydroxycholesterol induced mitochondria hyperpolarization (Fig. 5B) and the production of ROS (Fig. 5C).

Relationship between ROS levels and MTT activity

As part of the intracellular reduction of MTT has been associated with superoxide production (9), we determined if the production of ROS induced by oxLDL was associated with the increased MTT activity by MG-63 cells. As shown in Fig. 6A, incubation of cells with the antioxidant NAC prior to treatments with low concentrations of oxLDL did not prevent the increase in MTT activity. Moreover, depletion of cellular ROS scavenger glutathione by incubation with l-buthionine-(S,R)-sulfoximine (BSO), which increased basal and oxLDL-induced ROS levels (Fig. 6C), did not alter the basal MTT activity nor further increased the MTT activity induced by oxLDL in MG-63 cells (Fig. 6B), but rather reduced the MTT activity induced by oxLDL (two-way ANOVA, $P<0.001$).

Involvement of flavoenzyme and NAD(P)H on MTT activity

Both mitochondrial and non mitochondrial NAD(P)H- and flavoenzyme-dependent MTT activity have been reported (30). Therefore we hypothesized that the effect of oxLDL on MTT activity by MG-63 cells was associated with flavoenzyme activity and cellular NAD(P)H levels. As shown in Fig. 7A, the addition of flavoenzyme inhibitor DPI for 3 h reduced both basal (ANOVA, $P<0.003$; Dunnett's $P<0.05$ from 10μM) and oxLDL-induced MTT activity (ANOVA, $P<0.0001$; Dunnett's $P<0.01$ from 5μM) in a dose dependent manner. Therefore, our results indicate that DPI reduced in a comparable manner the MTT activity both in control cells as well as in cells treated with oxLDL which suggests that the latter increase the flavoenzyme activity. Moreover, NAD(P)H-associated cellular fluorescence was also increased by oxLDL (Fig. 7B) and the fluorescence specificity for NAD(P)H was demonstrated by the reduction of fluorescence with FCCP which promotes NADH oxidation.

Effect of oxLDL on the cellular amount of thiol containing proteins

As oxidative stress was shown to be induced by oxLDL in MG-63 cells (Fig. 5C), we postulated that mechanisms involving thiol-containing ROS scavenger proteins may be associated with the maintenance of cellular redox state. Members of the thiol-containing family of proteins undergo reversible oxidation/reduction catalyzed by proteins of the NADPH-dependent thioredoxin-fold family and thereby, contribute to maintain the redox state of cells (4). As shown in Fig. 8A, thiol content monitored by CMFDA fluorescence measurement was decreased in MG-63 cells following

incubation with oxLDL which indicates increased oxidation of thiol-containing proteins. Moreover, the expression of thiol-containing metallothionein (MT) in cells exposed to oxLDL was increased by 2.5-fold (Fig. 8B) which further suggests the induction of processes in order to maintain the redox state of cells.

The effects of low concentrations of oxLDL on osteoblastic functions

In order to determine the significance of the effects of low concentrations of oxLDL with respect to bone metabolism, we monitored osteoblastic functions under conditions of incubation with low concentrations of oxLDL. As exposure to oxLDL has been associated with increased osteoblastic proliferation with a concomitant reduction of differentiation (39), we determined the alkaline phosphatase activity in MG-63 cells exposed for 48 h to low concentrations of oxLDL. As shown in Fig. 9A, the treatment of MG-63 cells with low concentrations of oxLDL reduced the alkaline phosphatase activity indicating that osteoblastic functions are altered by low concentrations of oxLDL. In addition, we observed that 50 μg/ml of oxLDL reduced the basal migration of MG-63 cells whereas the same concentration of nLDL increased by 2.5-fold the migration (Fig. 9B).

DISCUSSION

Determination of hormesis-like effects induced by oxLDL and oxysterol

Incubation of osteoblastic cell lines with increasing concentrations of oxLDL and 7β-hydroxycholesterol, an oxysterol usually formed along copper-mediated LDL oxidation resulted in biphasic MTT activity dose-responses. Such non-monotonic dose-response relationship agrees with the qualitative characteristic of hormesis where low concentrations of a stressful stimulus trigger a stimulatory response, whereas an inhibitory response occurs at high concentrations (10). Furthermore, quantitative features of hormesis as established by Calabrese and Baldwin (9) we also observed: the average maximum amplitude of the stimulatory response was between 130-160% of the control, and the range of the hormetic zone was 10 to 20 fold. Moreover, the increase in MTT activity induced by oxLDL was time-dependent with a significant augmentation observed at 9h of incubation, a maximal effect occurring at 48 h that was not evident at 72h. Therefore we show for the first time that oxLDL and 7β-hydroxycholesterol induce hormesis-like effects in osteoblastic cells. Similar dose- and time-dependent induction of biphasic MTT activity responses by oxLDL have been reported in macrophage cells (16) according to their degree of oxidation. Moreover, similar hormesis-like response of MTT activity and time-dependent response have been shown for marrow stromal cells incubated with oxysterol cholestane-3b,5a,6b-triol (29).

Both direct stimulation hormesis (DSH) and overcompensation stimulation hormesis (OCSH) have been described with distinct temporal features (10). A disruption of homeostasis characterized as an initial reduction of end-point function monitored, a modest overcompensation, the reestablishment of homeostasis, and the adaptive nature of the process are all key conceptual features of OCSH. In contrast, DSH does not result from a disrupted homeostasis but represents a direct stimulatory response to initial stimuli, operating within normal maintenance functions without initial reduction of end-point function. In the current study, MTT activity was the endpoint function monitored and did not shown any initial reduction from 4 h. Therefore, our results more likely correspond to DSH.

Correspondence of MTT activity with cell proliferation and death

As the stimulatory MTT response trigger by low concentrations of oxLDL seems at a first glance associated to cell proliferation, we further determined the correspondence of the increased MTT activity with cell division. Our results indicate that part of the increase in MTT activity reflects a stimulation of osteoblastic cell proliferation by low concentrations of oxLDL. Accordingly, Parhami et al (39) have reported that minimally oxidized LDL particles promote cell proliferation and inhibit the differentiation of MC3T3 bone cells based on evidence of increased [^{3}H]-thymidine incorporation and inhibition of the induction of alkaline phosphatase as a marker for osteoblastic differentiation. On the other hand, our data affords arguments that suggest potential discrepancies between the increased MTT activity and cell

proliferation of MG-63 cells. First, a significant increase in MTT activity was seen as soon as 9h after the addition of oxLDL to the incubation media. Under high cell proliferation rate in the presence of serum, MG-63 cells rather showed doubling time of 28h (22). Therefore completion of cell cycle which would be associated with increased cell number and MTT activity within 9h is unlikely. Moreover, a ratio above 1 was shown when the relative MTT activity was normalized by relative cell number or cellular protein content. Therefore, part of the increased MTT activity by osteoblastic cells incubated with oxLDL was of other nature.

Our data also indicates that high concentrations of oxLDL particles promote an inhibitory response evidenced by the reduction of MG-63 cell viability, indicated by the loss of MTT activity and the reduction of cell number. Accordingly, we (6) and others (20, 29) have reported that oxLDL particles induce the apoptosis of osteoblastic cells followed by annexin V staining, DNA fragmentation, loss of lysosomal integrity and appearance of pro-apoptotic proteins. Furthermore, increasing concentrations of oxysterols such as 7β-hydroxycholesterol and 7-ketocholesterol resulted in the reduction of MG-63 cell viability (from 20-30 μM) as indicated by the loss of MTT activity after 48 h of incubation. Our results agree with studies of Liu et al. (29) which showed that concentrations above 15 μM of oxysterol cholestane-3b,5a,6b-triol promote cell death of primary rat bone marrow cells after 2 days of culture.

Oxidized LDL and lysosome activity

Given that a discrepancy was shown between MTT activity and cell proliferation for osteoblastic cells incubated with oxLDL, we further characterized the nature for oxLDL-induced increase of MTT activity. Inhibition of lysosomal activity by chloroquine and acidic compartment staining with acridine orange indicated that the increased osteoblastic MTT activity induced by oxLDL was not associated with enhanced endosomal/lysosomal activity. Nevertheless, we observed that incubation of MG-63 cells with low concentrations of oxLDL was associated with an increase of cell autofluorescence which suggest the presence of lipofuscin/ceroid-like materials. Lipofuscin/ceroid formation results from the progressive accumulation of biological "garbage" material, such as defective mitochondria, cytoplasmic protein aggregates as an intralysosomal undegradable material, with bright, wide-spectrum autofluorescence (8). It has been shown that after its uptake into macrophage lysosomes by receptor-mediated endocytosis, oxLDL particles are poorly degraded, resulting in ceroid-containing foam cells (27). Moreover, oxLDL-induced cytotoxicity in macrophages (26) and in osteoblast (6) has been associated with lysosomal rupture. Our results suggest for the first time that oxLDL particles may promote lipofuscin/ceroid accumulation in osteoblastic cells.

Effects of oxidized LDL on mitochondria of osteoblastic cells

We suspected that the increase of MTT activity could correspond to increased mitochondria mass or metabolic activity. Such an increase of mitochondrial mass under oxidative stress condition has been reported (24, 25). However our results

showed that the mitochondria mass was not increased by oxLDL and therefore can not account for the oxLDL-induced increase of MTT activity. However, low concentrations of oxLDL induced mitochondria membrane hyperpolarization in osteoblastic cells as has been reported in Caco-2 intestinal cells exposed to oxLDL (15). Mitochondrial hyperpolarization has been described as an early apoptotic event (34), and has been associated with an exponential increase in ROS production (21) being a major contributor to the oxidative signal induced by oxLDL (53). Accordingly, our results indicate that oxLDL particles stimulate the production of ROS in MG-63 cells. Therefore, mitochondrial hyperpolarization induced by low concentrations of oxLDL in osteoblastic cells may be part of the hormesis stimulatory response necessary to compensate and maintain the cellular metabolic homeostasis, disrupted by the stressful stimulus. However a higher metabolic rate is also associated with the production of ROS which may subsequently culminate in apoptosis. Since MTT activity has been associated with cellular superoxide production (9), the oxLDL-induced ROS production could result in the augmentation of MTT activity. However, the used of antioxidant NAC or BSO did not modify the oxLDL-induced increase of MTT activity in osteoblastic cells suggesting that the increase of MTT activity is not associated with intracellular ROS levels.

Association of the increased MTT activity with NAD(P)H-dependent mechanisms for the reduction of cellular thiols

As we report that oxLDL particles promote the production of ROS in osteoblastic cells, we suspected that pathways involved in ROS scavenging, such as thiol proteins, may be triggered. Accordingly, the cellular content of reduced thiol was decreased by exposure to oxLDL and the expression of thiol-containing metallothionein was increased. Moreover, the depletion of cellular ROS scavenger glutathione by incubation with BSO increased the levels of ROS induced by oxLDL and reduced the MTT activity induced by oxLDL suggesting that under these conditions the levels of ROS may not be regulated which lead to an accentuation of loss of cell viability. Flavoenzymes use flavin as coenzyme in a variety of electron transfer reactions required for energy producing, biosynthesis, and more particularly in detoxification and electron scavenging pathways. Key flavoenzymes in defence against oxidative stress are members of the thioredoxin-fold family of proteins (thioredoxin and glutoredoxin) which catalyzed the NADPH-dependent reduction of protein thiols in order to maintain the redox state of cells. Our results showed that oxLDL particles increase NAD(P)H cellular fluorescence. We also reported that the increased MTT activity induced by oxLDL was inhibited by the flavoenzyme inhibitor DPI. Therefore, oxLDL-induced increased MTT activity in osteoblastic cells may correspond to compensation mechanisms afforded by in order to maintain the redox state of cellular thiols.

OxLDL-induced hormesis in osteoblastic cells and bone metabolism

As shown by our results, induction of hormesis-like response by oxLDL in osteoblastic cells is associated with the stimulation of cell proliferation and ROS production by low concentrations of oxLDL. It is generally accepted that the stimulation of osteoblastic proliferation may compromise their differentiation into competent bone-forming cells (28, 38, 39). In accordance, our results indicate that low concentrations of oxLDL reduced the alkaline phosphatase activity, a marker of osteoblastic maturity. In addition, we showed that oxLDL compromise the migration of osteoblastic cells. Both functions have been shown to play a critical role in bone formation, remodeling and fracture repair (45). Therefore, our current study indicates that low concentrations of oxLDL may alter the bone metabolism by reducing osteoblastic differentiation in favour of uncontrolled cell proliferation and by affecting cell migration. On the other hand, high concentrations of oxLDL cause osteoblastic cell death that will result in reduced bone formation. In summary, our results indicate that oxLDL particles alter osteoblastic cell proliferation, migration and apoptosis rate, and thereby may contribute to alteration of bone metabolism equilibrium and may be responsible for the reduction of bone mass associated with atherogenic conditions.

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References

1. **Adami S, Braga V, Zamboni M, Gatti D, Rossini M, Bakri J, Battaglia E.** Relationship between lipids and bone mass in 2 cohorts of healthy women and men. *Calcif Tissue Int* 74:136-142, 2004.

2. **Banks LM, Lees B, MacSweeney JE, Stevenson JC.** Effect of degenerative spinal and aortic calcification on bone density measurements in post-menopausal women: links between osteoporosis and cardiovascular disease? *Eur J Clin Invest* 24:813-817, 1994.

3. **Barengolts EI, Berman M, Kukreja SC, Kouznetsova T, Lin C, Chomka EV.** Osteoporosis and coronary atherosclerosis in asymptomatic postmenopausal women. *Calcif Tissue Int* 62:209-213, 1998.

4. **Berndt C, Lillig CH, Holmgren A.** Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. *Am J Physiol Heart Circ Physiol* 292:H1227-H1236, 2007.

5. **Brissette L, Charest MC, Falstrault L.** Selective uptake of cholesteryl esters of low-density lipoproteins is mediated by the lipoprotein-binding site in HepG2 cells and is followed by the hydrolysis of cholesteryl esters. *Biochem J* 318 (Pt 3):841-847, 1996.

6. **Brodeur MR, Brissette L, Falstrault L, Ouellet P, Moreau R.** Influence of oxidized low-density lipoproteins (LDL) on the viability of osteoblastic cells. *Free Radic Biol Med* 44:506-517, 2008.

7. **Brown AJ, Jessup W.** Oxysterols and atherosclerosis. *Atherosclerosis* 142:1-28, 1999.

8. **Brunk UT, Terman A.** The mitochondrial-lysosomal axis theory of aging: accumulation of damaged mitochondria as a result of imperfect autophagocytosis. *Eur J Biochem* 269:1996-2002, 2002.

9. **Burdon RH, Gill V, Rice-Evans C.** Reduction of a tetrazolium salt and superoxide generation in human tumor cells (HeLa). *Free Radic Res Commun* 18:369-380, 1993.

10. **Calabrese EJ, Baldwin LA.** Defining hormesis. *Hum Exp Toxicol* 21:91-97, 2002.

11. **Calabrese EJ, Blain R.** The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. *Toxicol Appl Pharmacol* 202:289-301, 2005.

12. **Chen TL.** Inhibition of growth and differentiation of osteoprogenitors in mouse bone marrow stromal cell cultures by increased donor age and glucocorticoid treatment. *Bone* 35:83-95, 2004.

13. **Damelin LH, Alexander JJ.** Metal-induced hormesis requires cPKC-dependent glucose transport and lowered respiration. *Hum Exp Toxicol* 20:347-358, 2001.

14. **Damelin LH, Vokes S, Whitcutt JM, Damelin SB, Alexander JJ.** Hormesis: a stress response in cells exposed to low levels of heavy metals. *Hum Exp Toxicol* 19:420-430, 2000.

15. **Giovannini C, Matarrese P, Scazzocchio B, Sanchez M, Masella R, Malorni W.** Mitochondria hyperpolarization is an early event in oxidized low-density lipoprotein-induced apoptosis in Caco-2 intestinal cells. *FEBS Lett* 523:200-206, 2002.

16. **Han CY, Pak YK.** Oxidation-dependent effects of oxidized LDL: proliferation or cell death. *Exp Mol Med* 31:165-173, 1999.

17. **Holvoet P, Harris TB, Tracy RP, Verhamme P, Newman AB, Rubin SM, Simonsick EM, Colbert LH, Kritchevsky SB.** Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition study. *Arterioscler Thromb Vasc Biol* 23:1444-1448, 2003.

18. **Holvoet P, Jenny NS, Schreiner PJ, Tracy RP, Jacobs DR.** The relationship between oxidized LDL and other cardiovascular risk factors and subclinical

CVD in different ethnic groups: the Multi-Ethnic Study of Atherosclerosis (MESA). *Atherosclerosis* 194:245-252, 2007.

19. **Holvoet P, Van Cleemput J, Collen D, Vanhaecke J.** Oxidized low density lipoprotein is a prognostic marker of transplant-associated coronary artery disease. *Arterioscler Thromb Vasc Biol* 20:698-702, 2000.

20. **Klein BY, Rojansky N, Ben Yehuda A, Abou-Atta I, Abedat S, Friedman G.** Cell death in cultured human Saos2 osteoblasts exposed to low-density lipoprotein. *J Cell Biochem* 90:42-58, 2003.

21. **Korshunov SS, Skulachev VP, Starkov AA.** High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* 416:15-18, 1997.

22. **Labelle D, Jumarie C, Moreau R.** Capacitative calcium entry and proliferation of human osteoblast-like MG-63 cells. *Cell Prolif* 40:866-884, 2007.

23. **Lajeunesse D, Meyer RA, Jr., Hamel L.** Direct demonstration of a humorally-mediated inhibition of renal phosphate transport in the Hyp mouse. *Kidney Int* 50:1531-1538, 1996.

24. **Lee CF, Chen YC, Liu CY, Wei YH.** Involvement of protein kinase C delta in the alteration of mitochondrial mass in human cells under oxidative stress. *Free Radic Biol Med* 40:2136-2146, 2006.

25. **Lee HC, Yin PH, Lu CY, Chi CW, Wei YH.** Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. *Biochem J* 348 Pt 2:425-432, 2000.

26. **Li W, Yuan XM, Brunk UT.** OxLDL-induced macrophage cytotoxicity is mediated by lysosomal rupture and modified by intralysosomal redox-active iron. *Free Radic Res* 29:389-398, 1998.

27. **Li W, Yuan XM, Olsson AG, Brunk UT.** Uptake of oxidized LDL by macrophages results in partial lysosomal enzyme inactivation and relocation. *Arterioscler Thromb Vasc Biol* 18:177-184, 1998.

28. **Lian JB, Stein GS, Stein JL, van Wijnen AJ.** Transcriptional control of osteoblast differentiation. *Biochem Soc Trans* 26:14-21, 1998.

29. **Liu H, Yuan L, Xu S, Wang K, Zhang T.** Cholestane-3beta,5alpha,6beta-triol inhibits osteoblastic differentiation and promotes apoptosis of rat bone marrow stromal cells. *J Cell Biochem* 96:198-208, 2005.

30. **Liu Y, Peterson DA, Kimura H, Schubert D.** Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurochem* 69:581-593, 1997.

31. **Lougheed M, Steinbrecher UP.** Mechanism of uptake of copper-oxidized low density lipoprotein in macrophages is dependent on its extent of oxidation. *J Biol Chem* 271:11798-11805, 1996.

32. **Mackie EJ.** Osteoblasts: novel roles in orchestration of skeletal architecture. *Int J Biochem Cell Biol* 35:1301-1305, 2003.

33. **Maggio D, Barabani M, Pierandrei M, Polidori MC, Catani M, Moecocci P, Senin U, Pacifici R, Cherubini A.** Marked decrease in plasma antioxidants in aged osteoporotic women: results of a cross-sectional study. *J Clin Endocrinol Metab* 88:1523-1527, 2003.

34. **Matsuyama S, Llopis J, Deveraux QL, Tsien RY, Reed JC.** Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat Cell Biol* 2:318-325, 2000.

35. **Moreau R, Aubin R, Lapointe JY, Lajeunesse D.** Pharmacological and biochemical evidence for the regulation of osteocalcin secretion by potassium channels in human osteoblast-like MG-63 cells. *J Bone Miner Res* 12:1984-1992, 1997.

36. **Nyssen-Behets C, Duchesne PY, Dhem A.** Structural changes with aging in cortical bone of the human tibia. *Gerontology* 43:316-325, 1997.

37. **Orozco P.** Atherogenic lipid profile and elevated lipoprotein (a) are associated with lower bone mineral density in early postmenopausal overweight women. *Eur J Epidemiol* 19:1105-1112, 2004.

38. **Parhami F, Jackson SM, Tintut Y, Le V, Balucan JP, Territo M, Demer LL.** Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells. *J Bone Miner Res* 14:2067-2078, 1999.

39. **Parhami F, Morrow AD, Balucan JP, Leitinger N, Watson AD, Tintut Y, Berlinger JA, Demer LL.** Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients. *Arterioscler Thromb Vasc Biol* 17:680-687, 1997.

40. **Parhami F, Tintut Y, Beamer WG, Gharavi N, Goodman W, Demer LL.** Atherogenic high-fat diet reduces bone mineralization in mice. *J Bone Miner Res* 16:182-188, 2001.

41. **Poli A, Bruschi F, Cesana B, Rossi M, Paoletti R, Crosignani PG.** Plasma low-density lipoprotein cholesterol and bone mass densitometry in postmenopausal women. *Obstet Gynecol* 102:922-926, 2003.

42. **Ramseier E.** Untersuchungen uber arteriosklerotische veranderungen der knochenarterien. *Virchows Arch Path Anat* 336:77-86, 1962.

43. **Schmidt CM, Cheng CN, Marino A, Konsoula R, Barile FA.** Hormesis effect of trace metals on cultured normal and immortal human mammary cells. *Toxicol Ind Health* 20:57-68, 2004.

44. **Seibold S, Schurle D, Heinloth A, Wolf G, Wagner M, Galle J.** Oxidized LDL induces proliferation and hypertrophy in human umbilical vein endothelial cells via regulation of p27Kip1 expression: role of RhoA. *J Am Soc Nephrol* 15:3026-3034, 2004.

45. **Stains JP, Civitelli R.** Cell-to-cell interactions in bone. *Biochem Biophys Res Commun* 328:721-727, 2005.

46. **Steinberg D.** Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 272:20963-20966, 1997.

47. **Tintut Y, Morony S, Demer LL.** Hyperlipidemia promotes osteoclastic potential of bone marrow cells ex vivo. *Arterioscler Thromb Vasc Biol* 24:E6-E10, 2004.

48. **Uyama O, Yoshimoto Y, Yamamoto Y, Kawai A.** Bone changes and carotid atherosclerosis in postmenopausal women. *Stroke* 28:1730-1732, 1997.

49. **von der RP, Hansen MA, Hassager C.** The association between low bone mass at the menopause and cardiovascular mortality. *Am J Med* 106:273-278, 1999.

50. **Yamaguchi T, Sugimoto T, Yano S, Yamauchi M, Sowa H, Chen Q, Chilhara K.** Plasma lipids and osteoporosis in postmenopausal women. *Endocr J* 49:211-217, 2002.

51. **Yang P, He XQ, Peng L, Li AP, Wang XR, Zhou JW, Liu QZ.** The role of oxidative stress in hormesis induced by sodium arsenite in human embryo lung fibroblast (HELF) cellular proliferation model. *J Toxicol Environ Health A* 70:976-983, 2007.

52. **Zettler ME, Prociuk MA, Austria JA, Massaeli H, Zhong G, Pierce GN.** OxLDL stimulates cell proliferation through a general induction of cell cycle proteins. *Am J Physiol Heart Circ Physiol* 284:H644-H653, 2003.

53. **Zmijewski JW, Moellering DR, Le Goffe C, Landar A, Ramachandran A, Darley-Usmar VM.** Oxidized LDL induces mitochondrially associated reactive oxygen/nitrogen species formation in endothelial cells. *Am J Physiol Heart Circ Physiol* 289:H852-H861, 2005.

Legend to the figures

Fig. 1. **Effects of oxidized LDL on MTT activity by osteoblastic cells.** (A) Cells were incubated for 48h with increasing concentrations of native LDL, oxLDL or culture medium. MTT activity was determined as described in the Material and methods section. B) Cells were incubated for different time intervals with 20 μg/ml of oxLDL and the MTT activity was determined. Data are expressed as the relative MTT activity (mean ± SEM) compared to control conditions without lipoprotein from at least 3 independent experiments performed in tetraplicate.

Fig. 2. **Effects of oxysterols on MG-63 cells.** A) Cells were incubated for 48 h with increasing concentrations of 7β-hydroxycholesterol or 7-ketocholesterol and MTT activity were determined. B) Cells were incubated for different intervals of time with 10μM of 7-ketocholesterol (7-ketoChol) or 7β-hydroxycholesterol (7OHChol) and the MTT activity was determined. Data are expressed as the relative MTT activity (mean ± SEM) compared to control conditions without oxysterol from at least 3 independent experiments performed in triplicates.

Fig. 3. **Effects of oxidized LDL on cell division of MG-63.** Cells were incubated either with 200μg/ml of nLDL, various concentrations (μg/ml) of oxLDL or in culture medium (0 μg/ml) for 48h. Thereafter, cell numbers (A) were

determined by flow cytometer and cell division (B) was followed by the decrease in CFSE fluorescence as described in the Material and Methods section. Data represent means ± SEM for 6 to 8 independent experiments expressed as the relative cell numbers compared to control condition or the relative cell division (1/ratio of CFSE fluorescence of treated vs control cells). Basal cell number prior to the 48h incubation period is shown. One-way ANOVA, Dunnett's: $^{£}P<0.05$ and $^{££}P<0.01$ for significant reduction compared to the control condition without lipoprotein, $*P<0.05$ and $**P<0.01$ for significant increase compared to the control condition without lipoprotein; Two-tailed Student t test: $^{\#}P<0.01$, significant increase compared to the control condition without lipoprotein. C and D) Relative MTT activities were normalized according to relative cellular protein content or relative cell number respectively. Data are the mean ± SEM from at least 3 independent experiments. One-way ANOVA, Dunnett's: $*P<0.05$ and $**P<0.01$ for significant increase compared to the control condition without lipoprotein.

Fig. 4. **Determination of lysosomal-dependent MTT activity.** A) Cells were incubated in culture medium (CTL) or with 20µg/ml of oxLDL for 48h. *Left panel* shows representative cellular autofluorescence monitored as described in the Materials and methods section with the corresponding phase contrast image. *Right panel:* Data are expressed as the mean ± SEM of the relative autofluorescence compared to control condition from at least 3 independent

experiments. One-way ANOVA, Dunnett's: *P<0.05, **P<0.01, ***P<0.001 compared to control condition without lipoprotein. B) Cells were incubated in culture medium (CTL) or with 20μg/ml of oxLDL for 48h and acridine orange staining was performed (left panel: representative image). *Right panel*: cells were incubated in culture medium (0 μg/ml) or with 20μg/ml of oxLDL for 24h. Subsequently, cells were preincubated with increasing concentrations (μM) of chloroquine for 1h and thereafter MTT activity was determined thereafter as described in the Materials and methods section. Data are expressed as the mean ± SEM of the relative MTT activity compared to control condition without lipoprotein from at least 3 independent experiments. Two-tailed Student t test: **P<0.01, ***P<0.001 compared to control condition without lipoprotein.

Fig. 5. **Effects of oxidized LDL on mitochondria mass, membrane potential and ROS production in MG-63 cells.** A) Cells were incubated in culture medium (0 μg/ml)), 200μg/ml of nLDL or increasing concentrations (μg/ml) of oxLDL for 48h. Thereafter cells were analysed by flow cytometry for determination of the mitochondria mass using the MitoTracker green. Data are the mean ± SEM expressed as the relative mitochondria mass compared to control condition from 5 individual experiments. (B) Cells were incubated with 200μg/ml of nLDL, with various concentrations of oxLDL (μg/ml) or 7β-hydroxycholesterol (7βOHChol, μM), or in culture medium (0μg/ml) for

24 h and measurements of mitochondria membrane potential by JC-1 was performed as described in the Material and methods section. Incubation for 30 min with FCCP (5μM) was used as positive control for mitochondria membrane depolarisation. Data are the mean ± SEM expressed as the relative JC-1 red vs green fluorescence ratio from at least 3 individual experiments. One-way ANOVA, Dunnett's: *P<0.05, **P<0.01 for increased ratio compared to control condition; Two-tailed Student t test: $^{£}$P<0.05 for reduced ratio compared to control condition. C) *Left:* Cells were incubated with 200μg/ml of nLDL, with various concentrations of oxLDL (μg/ml) or 10 μM 7β-hydroxycholesterol (7βOHChol), or in culture medium (0μg/ml) for 1, 3 and 24 h and the ROS levels were determined by CM-H2DCF-DA fluorescence. Data are the mean ± SEM expressed as the relative fluorescence vs control condition from at least 3 individual experiments. *P<0.05, one-way ANOVA, Dunnett's compared to control condition; **P<0.01, ***P<0.001, two-tailed Student t test compared to control condition. *Right*: representative data of CM-H2DCF-DA fluorescence (FL1-H) for cells incubated in culture medium (CTL) or with 20μg/ml of oxLDL for 1h.

Fig. 6. **Relationship between MTT activity and the intracellular levels of ROS.** A and B) Cells were incubated for 24h with 1mM NAC or 10 μM BSO prior to treatments with 10, 20 and 50μg/ml of oxLDL or in culture medium (0μg/ml) in the presence of NAC or BSO for 48h. MTT activity was determined as

described in the Material and methods section. The data is expressed as the ratio of MTT activity compared to control conditions (mean ± SEM) from at least 3 independent experiments performed in triplicates. One-way ANOVA, Dunnett's: ***P<0.001 compared to the corresponding conditions without lipoprotein. C) Cells were incubated for 24 h without (CTL) or with 10μM BSO prior to treatments without (0 μg/ml) or with various concentrations (μg/ml) of oxLDL for 1 and 3. The ROS levels were determined by CM-H2DCF-DA fluorescence as described in Materials and methods section. Data represents means ± SEM expressed as the relative fluorescence vs control condition from at least 3 individual experiments. Two-tailed Student t test: *P<0.01 compared to control condition without BSO, One-way ANOVA, Dunnett's: $^{\#\#}$P<0.01 compared to condition without lipoprotein.

Fig. 7. **Association of flavoenzymes and NAD(P)H with MTT activity.** A) Cells were incubated without (0 μg/ml) or with various concentrations of oxLDL for 24 h. Subsequently, cells were preincubated with increasing concentrations (μM) of the flavoenzyme inhibitor DPI for 1h and thereafter MTT activity was determined as described in the Materials and methods section. Data are expressed as means ± SEM of the relative MTT activity compared to the control condition without lipoprotein from at least 3 independent experiments. Two-tailed Student t test: ***P<0.001 compared to the control condition without lipoprotein. B) Cells were incubated in culture

medium (CTL) or with 10 or 20 µg/ml of oxLDL for 48 h. Images are representative NAD(P)H-associated fluorescences analysed as described in the materials and methods section. Incubation with 5µM FCCP for 40 min prior to analysis of fluorescence was performed to determine the fluorescence specificity for NAD(P)H.

Fig. 8. **Evaluation of the content of thiol-containing proteins in cells exposed to oxLDL.** A) Cells were incubated in culture medium (CTL) or in the presence of 200 µg/ml nLDL or 20 µg/ml oxLDL for 24 h. Thereafter, cells were loaded with CMFDA and the fluorescence were analysed by confocal microscopy and values are expressed as means ± SEM of the relative CMFDA fluorescence compared to values of the control conditions from at least 3 independent experiments. Two-tailed Student t test: **$P<0.01$ compared to the control condition. B) Cells were incubated in culture medium (CTL) or in the presence of 20 µg/ml oxLDL for 24 h. Total RNA was isolated and subjected to RT-PCR using specific primers for human metallothionein 1/2 (MT) and GAPDH. Densitometric determinations were analysed and expressed as the relative MT expression normalized against GAPDH expression when compared to the control condition of 3 independent experiments. Two-tailed Student t test: *$P<0.05$.

Fig. 9. **Effect of low concentrations of oxLDL on osteoblastic functions.** A) Cells were incubated in culture medium (CTL) or in the presence of nLDL or oxLDL

for 48 h. Then, measurements of alkaline phosphatase activity (ALPase) by cellular protein extracts were performed as described in the Materials and Methods section. The data are expressed as means ± SEM of the relative ALPase compared to the control condition of 3 independent experiments. Two-tailed Student t test: **P<0.01 compared to the control condition. B) Cells were grown to confluent monolayer and were wounded by scratching the surface. This initial wounding (0 h) and the movement of the cells in the scratched area after 24 h were photographically monitored. The number of cells that have migrated to the wounded area was determined using imageJ software. The values are expressed as means ± SEM of the relative cell migration compared to control condition in the culture medium of 3 independent experiments. Two-tailed Student t test: **P<0.01, significant increase compared to the control condition; $^{£}$P<0.05, significant reduction compared to the control condition.

Figure 1. Hamel et al.

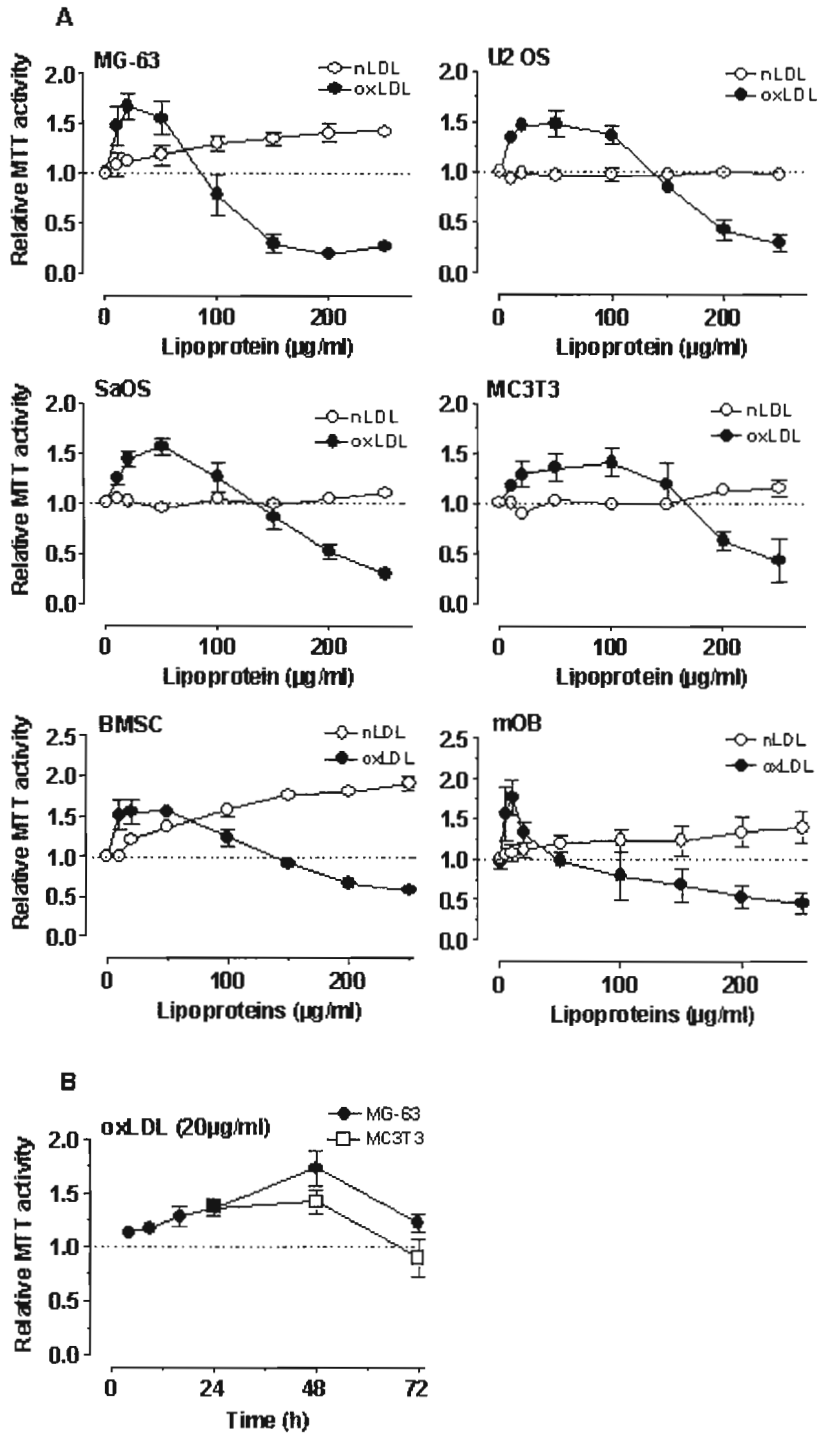


Figure 2. Hamel et al.

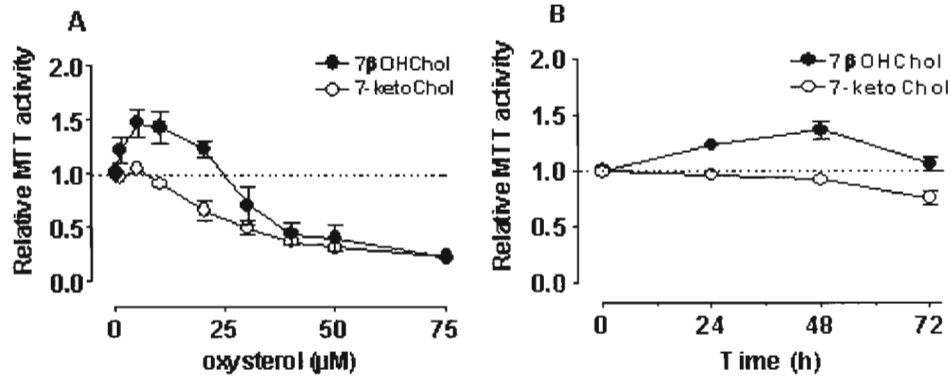
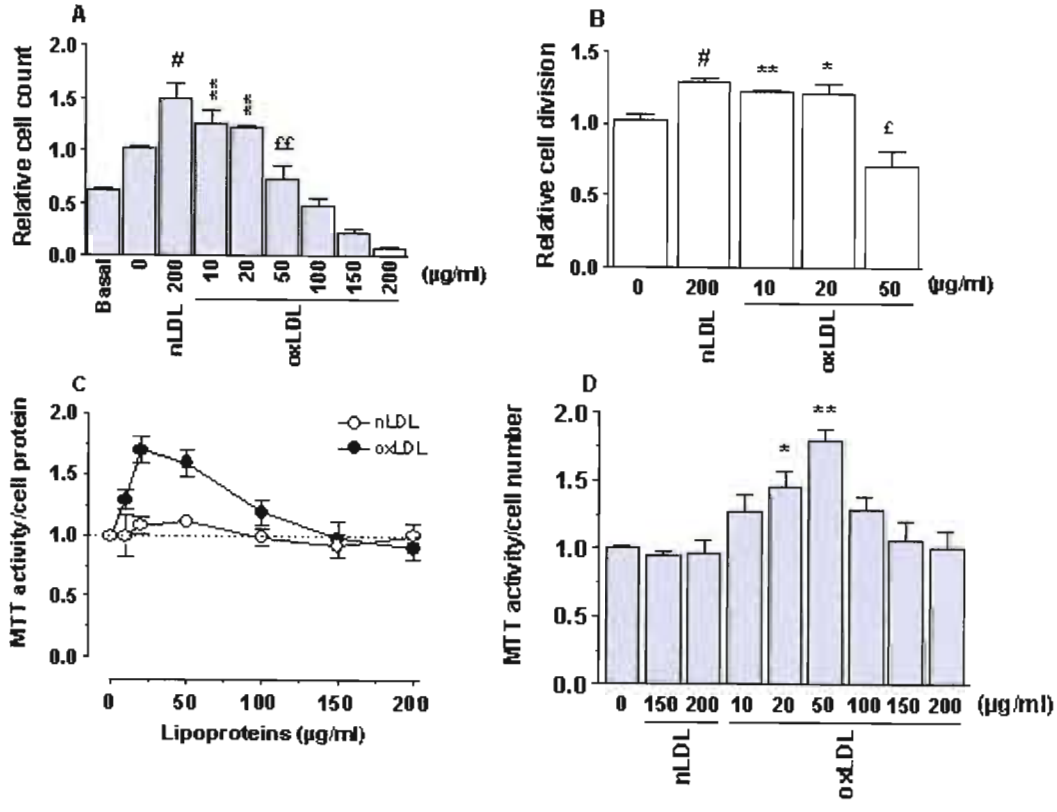


Figure 3. Hamel et al.



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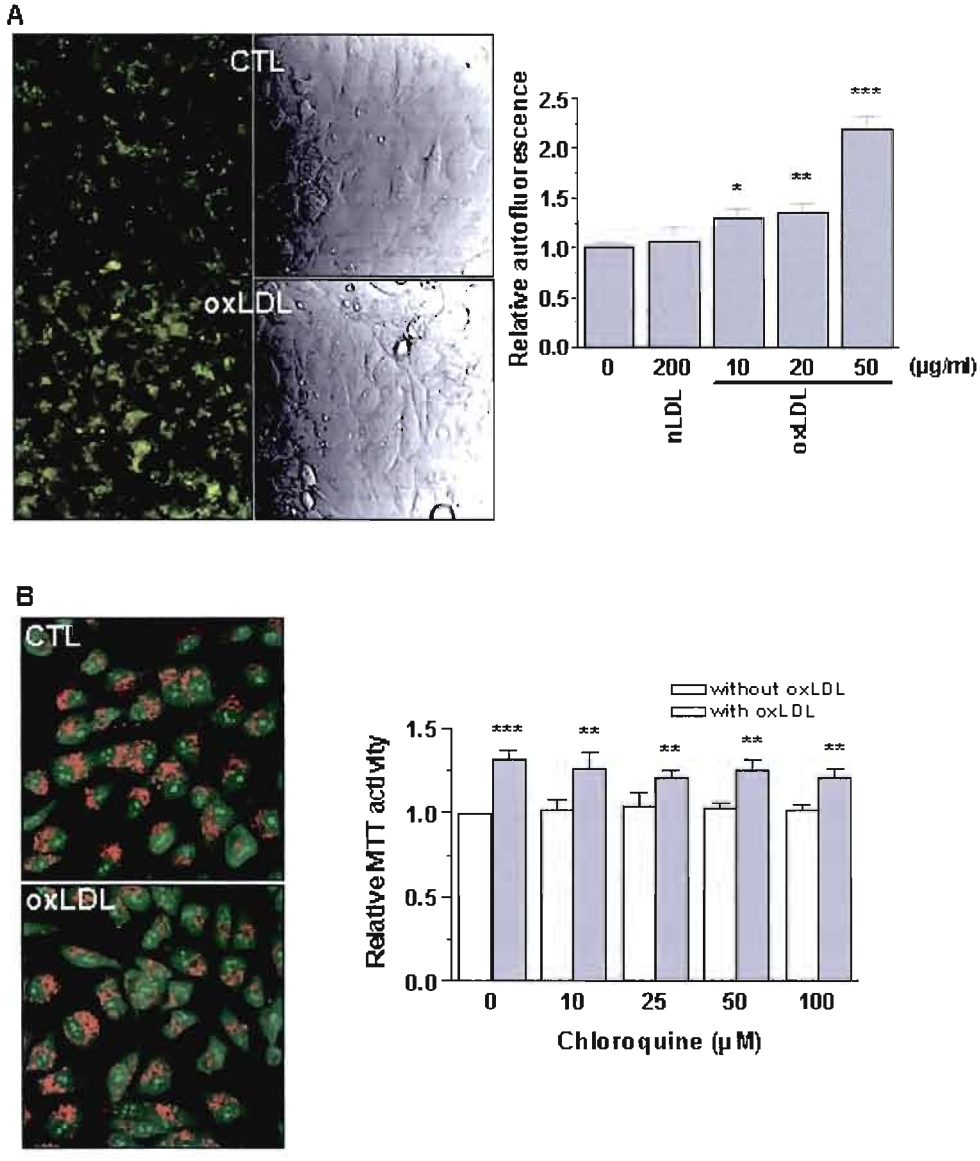

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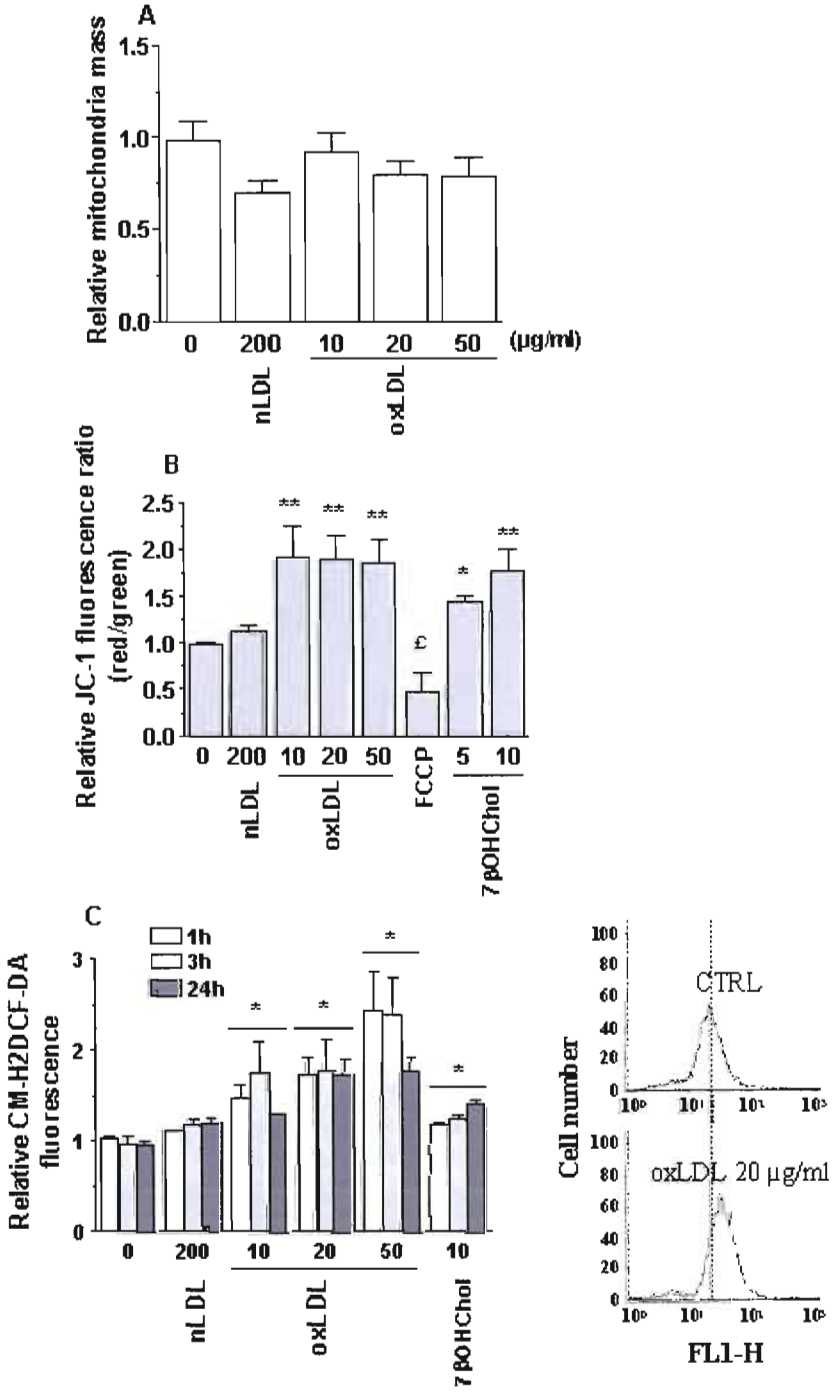


Figure 6. Hamel et al.

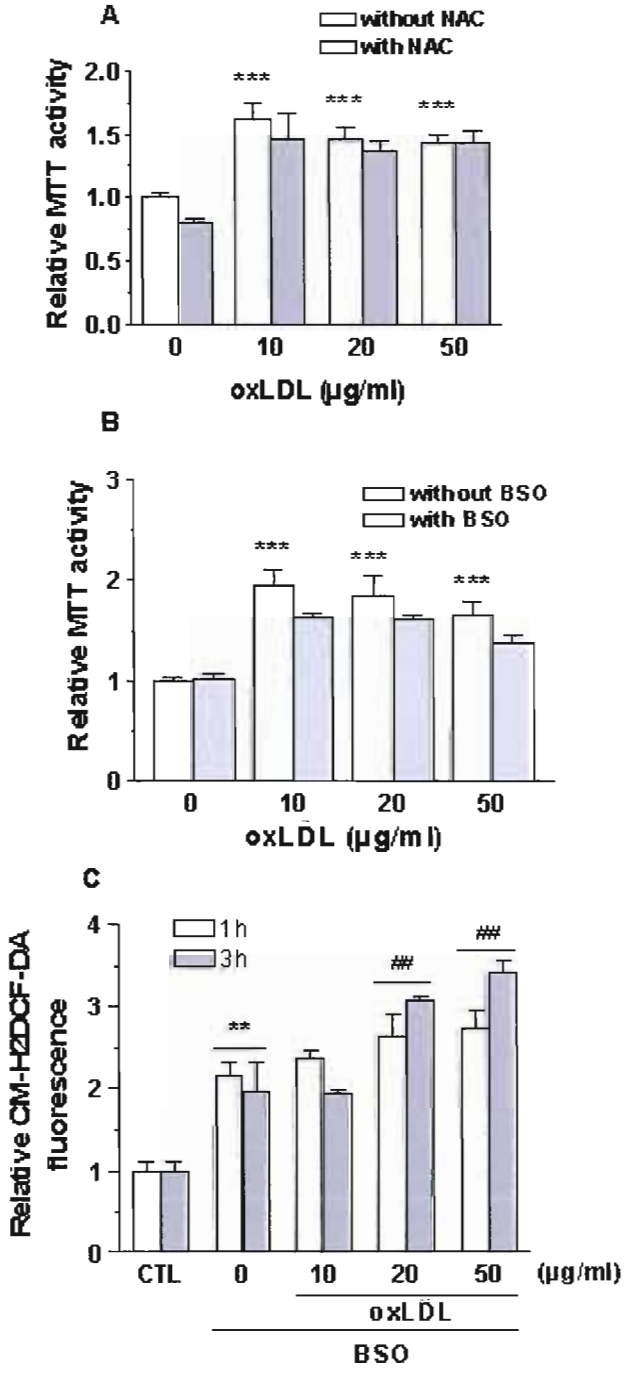


Figure 7. Hamel et al.

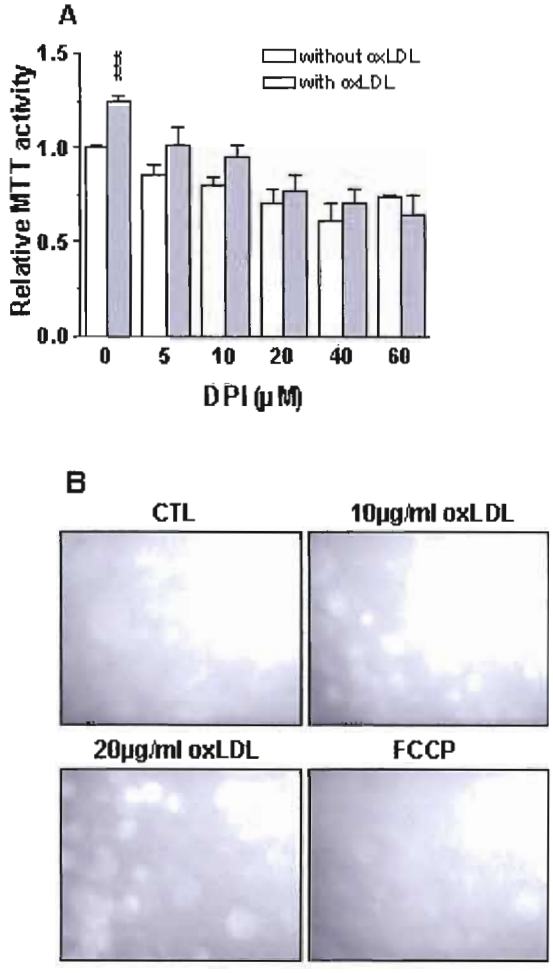


Figure 8. Hamel et al.

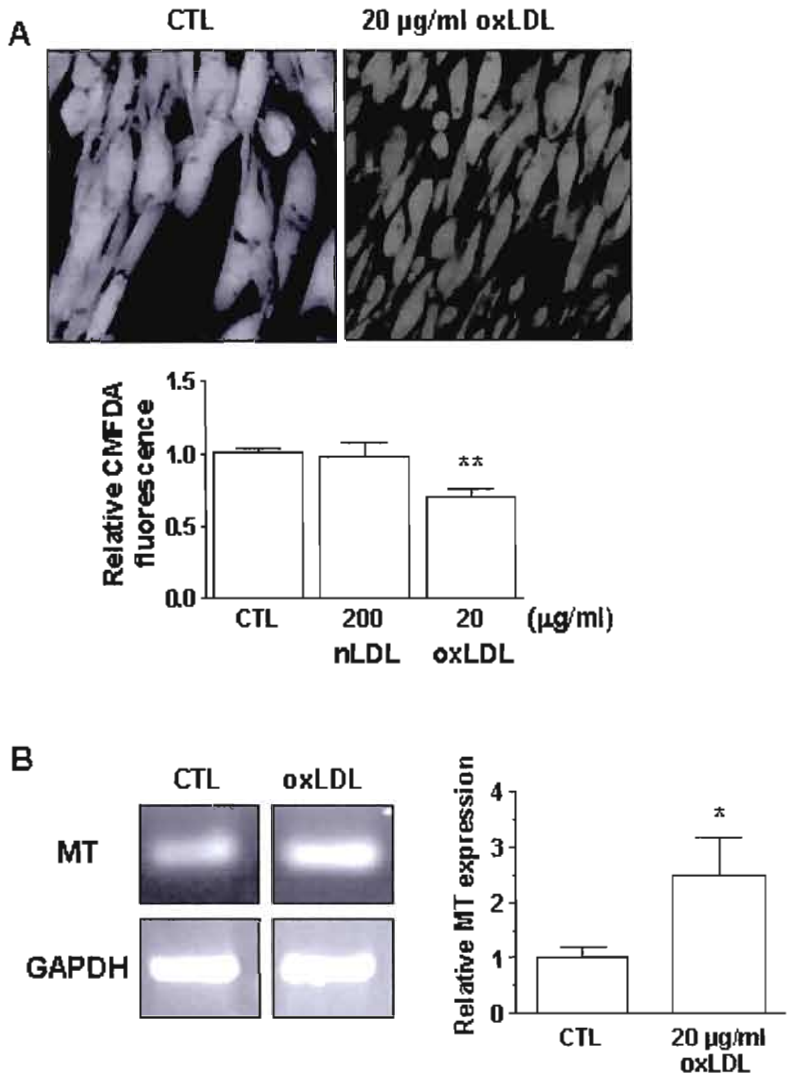
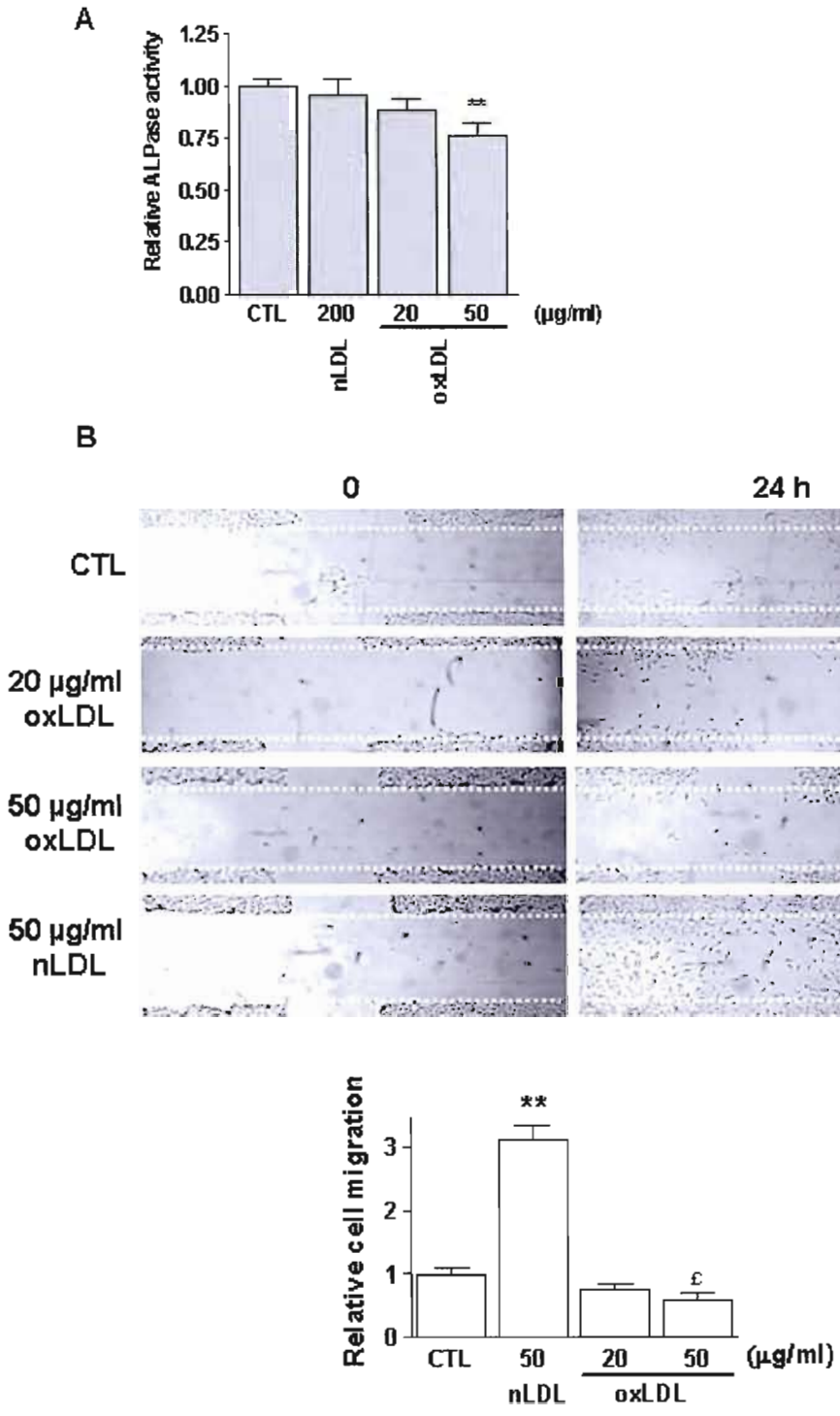


Figure 9. Hamel et al.



APPENDICE B

INVOLVEMENT OF TRANSIENT RECEPTOR POTENTIAL 7 (TRPM7) CHANNELS IN CADMIUM UPTAKE AND CYTOTOXICITY IN MC3T3 OSTEOBLASTS

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Abstract

Exposure to cadmium (Cd) disrupts bone metabolism, causing osteoporosis. Impaired vitamin D metabolism was initially proposed as the underlying mechanism, yet recent studies argue for the direct effect of Cd on bone cells. This study aimed at characterizing Cd uptake and cytotoxicity in MC3T3 osteoblasts. Mitochondrial activity and ^{109}Cd uptake were estimated as a function of extracellular concentrations of calcium (Ca) or magnesium (Mg). Time-dependent accumulation of ^{109}Cd was observed with a 50% lethal concentration (LC_{50}) of 9.0 ± 1.3 μM at 24-h. Extracellular Ca or Mg both decreased ^{109}Cd uptake and cytotoxicity. Cellular Mg starvation, a condition known to activate TRPM7 channels, enhanced ^{109}Cd uptake. Silencing TRPM7 channel expression abolished the Mg-sensitive and the Mg starvation-induced uptake suggesting that TRPM7 is involved in Cd transport in osteoblasts. We also show that bone-like mineralized matrix generated by differentiated MC3T3 cells accounts for significant Cd adsorption leading to reduced cytotoxicity but also represents a sink for metal storage.

Keywords: osteoblasts; cadmium; cytotoxicity; calcium; magnesium; transient receptor potential 7 channel; bone-like extracellular matrix

1. Introduction

Cadmium (Cd) is a divalent toxic transition metal and its use in the manufacturing of fungicides and batteries created significant industrial sources of emission (1). Chronic low-level exposures to Cd have been linked to increased risks of bone fracture (2). Cd may disrupt bone metabolism by indirect and direct mechanisms of toxicity. In indirect toxicity, circulating Cd affects renal proximal tubular cells, leading to decreased vitamin D_3 activation thus impairing intestinal Ca^{2+} absorption and subsequent bone remodelling (3,4). Direct toxicity implies that non nephrotoxic low Cd levels in the blood stream reach the bone compartment to exert its toxic effects on cells (5,6).

Bone metabolism is mainly regulated by two cell types (7): osteoblasts differentiating from mesenchymal progenitors (MPCs) that secrete and mineralize the extracellular matrix (ECM), and osteoclasts originating from the fusion of hematopoietic precursors who degrade the old or damaged bone. The balance between bone formation and resorption is critical for adequate remodelling of the skeleton; any unbalance leads to symptoms ranging from osteoporosis (bone mass deficit) to osteopetrosis (excessive bone mass) (8). Differentiation, renewal rate and lifespan of osteoblasts are crucial elements for bone remodelling (8).

A key parameter in cytotoxicity is cellular accumulation; little data is available about the uptake mechanisms involved in Cd accumulation in osteoblasts. We have shown that Cd accumulation in human osteoblast-like MG-63 cells is inhibited by Ca and

Mg although being voltage-insensitive (9) suggesting that osteoblastic uptake of Cd may involve non selective cation channels. The "transient receptor potential" (TRP) channels form a superfamily of cationic transporters (10) subdivided into three main families: TRPVs (Vanilloids), TRPMs (Melastatin) and TRPCs (Canonical). Expression of the "TRP-melastatin-related 7" (TRPM7) channel, which is permeable to Ca^{2+}, Mg^{2+} and Cd^{2+} ions (11), has been demonstrated in osteoblasts (12). We therefore characterized Cd uptake and toxicity in the murine MC3T3 osteoblast cells with emphasis on the involvement of TRPM7. Moreover, this cell model allows studying the influence of osteoblast differentiation on Cd accumulation and cytotoxicity.

2. Materials and methods

2.1 Cell culture. MC3T3-E1 (American Type Culture Collection, ATCC; Rockville, MD, USA) was maintained in alpha-modified minimum essential medium (alpha-MEM; Sigma, Oakville, Ontario, Canada) and subcultured every 7 days with 0.05% trypsin-0.02% EDTA solution (Invitrogen) as described previously (12). At confluence (designated D0), generally 7 days post-seeding at a 15 000 cells/cm^2 density, cells were induced to differentiate by incubation in a modified MEM medium (HyClone, Logan, UT, USA) containing 10% FBS, 10 mM β-glycerophosphate (Sigma) and 50 μg/mL ascorbic acid (Sigma) during an additional 28-days culture period (designated D28T *vs.* D28B). To assess osteoblastic differentiation, cells grown in 24-well plates (Starstedt, Montréal, Quebec, Canada) were solubilised in ice-cold buffer (100 mM glycine, 1 mM $MgCl_2$, 0.5% Triton X-100, pH 10.5) for alkaline phosphatase (ALP) activity determination (conversion of *p*-nitrophenylphosphate (*p*-NPP; Sigma) into *p*-nitrophenolate (*p*-NP) as described previously (13)) or fixed in 70% ice-cold ethanol for mineralization quantification. Mineralization was estimated following incubation in Alizarin Red S (ARS : 40 mM in a 10% (w/v) cetylpyridinium chloride [CPC] buffer dissolved in 10 mM Na_2PO_4, pH 4.1; all from Sigma). Cells were washed with ultrapure water, the Ca-ARS complexes were solubilised in 10% CPC buffer and the absorbance was read at 575 nm. The values are expressed as percentage of mineralization relative to D0 control cells cultured in MEM with 10% FBS.

2.2. Cytotoxicity assays. Cell viability was measured using the microtiter tetrazolium (MTT) method (9). Cells were seeded in 96-well plates (Sarstedt) and serum-starved 24 h prior to exposure to increasing concentrations of Cd in phenol- and serum-free DME/F12 (purchased as Ca- and Mg-free medium) with the addition of desired levels of Ca and Mg. The LC_{50} (50% lethal concentration) values were estimated in the presence of 0.1-1 mM Ca, 0-0.8 mM Mg or 0-100 μM 2-APB, as well as in the presence of 0-100 μM Bay K8644 or 0-100 μM Verapamil for the stimulation and the inhibition of voltage-dependent Ca^{2+} channels, respectively, or 30 μM SKF96365 and 5 μM Thapsigargine (TG), for the inhibition and the stimulation of TRPC channels, respectively (all from Sigma).

2.3. Cd accumulation measurements. Accumulation measurements were conducted as described previously (9) in chloride (Cl^-) or nitrate (NO_3^-) medium (used to increase the concentration of Cd^{2+} over chloro-complexes formation (14)) or in serum-free DME/F12. The cells were exposed to 0.5 μM ^{109}Cd (0.3 μCi/nmol; Eckert & Ziegler, Berlin, Germany) for 3 min or 24 h and accumulation levels were measured in the presence of 5 mM Ca, 2.5 mM Mg, 0-100 μM 2-APB, 0-100 μM Bay K8644, 0-100 μM Verapamil, 5 μM TG or 30 μM SKF96365. Results are expressed as pmol of ^{109}Cd per mg protein or per dish. The kinetic parameters of ^{109}Cd uptake were analyzed according to the modified Michaelis-Menten as described previously (9).

2.4. Silencing of TRPM7 gene. For silencing of TRPM7 gene, cells were transfected for 48 h with small interfering RNA (siRNA) targeting the TRPM7 mRNA sequence ATGGAATTTAAAGAAGTA (siTRPM7), as well as a nontargeting negative control siRNA (siRNA(-)) as described previously (12). The medium was changed for Mg-free DME/F12 for the last 24 h of incubation where specified, and the cells were used for ^{109}Cd transport measurements.

2.5. RNA extraction and semi-quantitative RT-PCR. Total RNA was extracted using the TriZol reagent (Invitrogen). Two μg of total RNA were reversed transcribed using the QIAGEN RT kit, followed by a 40 cycle PCR (Labnet Multigene II) at a 58°C annealing temperature using the QIAGEN PCR kit with specific primers for GAPDH and TRPM7 (12), as well as for osteocalcin (OCN), an osteoblast differentiation marker (F: 5'-CAAGTCCCACACAGCAGCTT-3', R: 5'-AAAGCCGAGCTGCCAGAGTT-3'). The PCR products were visualized with ethydium bromide staining following an electrophoresis on 2% agarose gel.

2.6. Measurement of Cd adsorption on ECM and Col I. Adsorption of Cd to D0- and D28-cell-secreted ECM was estimated following hypotonic shock by incubation in ultrapure water until complete cell lysis. Remaining ECM in the dishes were rinsed with Cl^- medium, and then exposed for 3 min to ^{109}Cd-labeled NO_3^- medium. In parallel, dishes were incubated for 2 h at 37 ^0C with 1mL rat tail type I collagen (Col I) (2 mg/mL) (kind gift from Dr. Annabi, UQAM, Montréal, Canada), then dried for 24 h under UV irradiation prior to a 3-min exposure to ^{109}Cd-labeled NO_3^- medium.

Competition experiments were conducted by exposing the ECM or Col I-coated dishes to the radiotracer-containing NO_3^- medium in the presence of 100 μM unlabeled Cd.

2.7. Statistical analysis. Nonlinear regression analyses were performed with Prism 4 software (GraphPad Software, San Diego, California, USA). The errors associated with the parameter values given in the text represent the standard error of regression (SER). Statistical analyses were performed with the unpaired student's *t*-test or ANOVA with Dunnett's or Bonferoni's post-test.

3. Results and discussion

3.1. Characterization of Cd uptake and cytotoxicity in MC3T3 cells

Our recent study revealed that Cd accumulation in human osteoblast-like MG-63 cells may involve non selective Ca^{2+}/Mg^{2+} channels (9) which remained to be identified. Further investigations were undertaken using murine osteoblast MC3T3 cell line which allows studying the influence of osteoblast differentiation on Cd accumulation and cytotoxicity. Cells were exposed to 0.5 μM ^{109}Cd in the culture medium (Fig. 1A). Cd accumulation was time-dependent and reached about 475 pmol. mg^{-1} protein at 24 h, a level quite comparable to MG 63 cells (9). A loss in cell viability was observed following a 24-h exposure to increasing concentrations of Cd (Fig. 1B). Data analyses gave an LC_{50} value of 9.0 ± 1.3 μM similar to that estimated in the human hepatoma cell line HepG2 (15), as well as in the pituitary cell line GH_4C_1 (16) but lower compared to the LC_{50} estimated in MG-63 cells (9). Figure 1C shows inhibition of the 3-min cellular uptake of 0.5 μM ^{109}Cd in the NO_3^- medium by increasing concentrations of unlabeled Cd demonstrating the involvement of a specific system of transport for Cd uptake. Data analyses gave the following kinetic parameter values: K_m = 3.3 ± 0.6 μM; V_{max} = 136 ± 21 pmol $^{109}Cd.3\ min^{-1}.mg\ protein^{-1}$; k_D = 28 ± 1 pmol $^{109}Cd.3\ min^{-1}.\mu M^{-1}.mg^{-1}$ protein. Note that k_D represents all non specific contributions to total uptake levels. Total cellular accumulation values in subsequent experiments have been corrected for the non specific contribution estimated in the presence of an excess of 100 μM unlabeled Cd. The K_m

value is similar to that reported in primary cultures of rat hepatocytes (17) and human intestinal crypt HIEC cells (18), but lower than the value reported for MG-63 cells (9). The V_{max} value obtained in MC3T3 cells is somewhat lower than those evaluated in rat hepatocytes, HIEC and MG-63 cells (9). Cd uptake was also found to vary with metal speciation since conditions known to increase the level of Cd^{2+} species (NO_3^- *vs.* Cl^- medium) significantly enhanced uptake levels (data not shown). Preferential uptake of Cd^{2+} species has also been observed in the Caco-2 (14) and MG-63 cells (9), suggesting the involvement of cationic transport mechanisms for Cd^{2+} uptake.

3.2. Impact of Ca and Mg on the cytotoxicity and uptake of Cd

Ca^{2+} and Mg^{2+} are essential ions to several cell functions and numerous channels allow their passage across the cell membrane (19,20). Cd being a toxic metal, it is generally accepted that it enters the cell via transport mechanisms devoted to essential metals. We hypothesized that Ca^{2+} may compete with Cd^{2+} for cellular uptake, therefore influencing Cd accumulation and cytotoxicity. MC3T3 cells sensitivity to Cd increased significantly when Ca concentration was lowered from 1 to 0.1 mM (Fig. 2A; LC_{50} values of 10.3 ± 1.1 *vs.* 5.1 ± 1.1 μM, respectively, $p<0.001$; 2-way ANOVA). The 24-h accumulation of ^{109}Cd decreased upon addition of 5 mM Ca in the culture medium (Fig. 2B) and the 3-min specific uptake measured in the nitrate medium was inhibited by half (Fig. 2C). Lowering Ca levels alone did not modify cell viability (data not shown). Therefore, Ca^{2+} and the cationic metal ion Cd^{2+} may, at least in part, share common transport mechanisms. We have

demonstrated that voltage-dependent Ca^{2+} channels (VDCCs) were not responsible for Cd uptake in MG 63 cells (9); similar observations were made for MC3T3 cells (data not shown). Therefore, other Ca uptake mechanisms were tested for their ability to transport Cd in MC3T3 cells. As we have reported for MG-63 cells (9), TG or SKF96365 were without effect on cytotoxicity and accumulation of Cd in MC3T3 cells (data not shown). We investigated the involvement of other Mg^{2+}/Ca^{2+} channels, TRPM7 (21,22), which are expressed in MC3T3 cells (23). Variations in extracellular Mg concentration did not affect cell viability (data not shown) but LC_{50} values in response to exposure to Cd decreased in the absence of Mg in the culture medium (LC_{50} values of 10.3 ± 1.1 *vs.* 5.1 ± 1.1 μM; Fig. 2A). Also, the 24-h accumulation of Cd was reduced when Mg concentration in the culture medium increased from 0.8 to 2.5 mM (Fig. 2B). Moreover, 2.5 mM Mg inhibited by 40% the 3-min specific uptake of 0.5 μM ^{109}Cd in the nitrate medium (Fig. 2C). The Mg-sensitive part of Cd uptake was further investigated in the presence of 2-APB which inhibits TRPMs in addition to TRPCs (24,25). Fig. 2D shows that 100 μM 2-APB inhibited Cd uptake under Mg-free exposure conditions and to a similar proportion as 2.5 mM Mg (Fig. 2C). Moreover, 2-APB did not inhibit Cd uptake in the presence of Mg (data not shown); no additive effects were observed between Mg- and 2-APB-mediated inhibitions suggesting that both act on similar uptake pathways. Altogether, these results suggest that TRPM Mg^{2+}/Ca^{2+} channels, namely TRPM7, may be involved in Cd^{2+} uptake in MC3T3 cells.

3.3. Impact of TRPM7 gene silencing

To directly test whether TRPM7 is involved, ^{109}Cd uptake was measured in cells transfected with small interfering RNAs (siRNA) targeting TRPM7 channels. Silencing TRPM7 gene (Fig. 3A) did not inhibit Cd uptake in Mg-free nitrate medium but completely abolished the Mg-sensitive component (Fig. 3B). Since low extracellular concentrations of Mg was shown to activate TRPM7 in osteoblasts (23), ^{109}Cd uptake was measured in cells maintained for 24 h under low Mg culture conditions. ^{109}Cd uptake significantly increased in Mg-starved control or siRNA(-) but not siTRPM7-transfected cells (Fig. 3B). Since Mg deprivation did not modify TRPM7 expression (Fig. 3A), the observed increase in ^{109}Cd uptake is likely the result of low Mg-induced activation of TRPM7 channels. Thus TRPM7 may be responsible of part of the specific uptake of the cationic metal ion Cd^{2+} in MC3T3 cells and condition known to activate TRPM7 lead to a preponderant involvement of TRPM7 in Cd uptake.

3.4. Cd uptake and cytotoxicity as a function of osteoblastic differentiation status

To investigate the influence of osteoblast differentiation on the cellular accumulation and cytotoxicity of Cd, MC3T3 cells were induced to differentiate (26). Cells treatment increased ALP activity (Fig. 4A) as well as OCN mRNA levels (data not shown), two osteoblastic differentiation markers (26,27). Cells sensitivity to Cd decreased as a function of the differentiation status (Fig. 4B). To note, at confluence

(D0), cellular proliferation stops and cells secrete significant quantities of ECM; in D28 cell cultures, protein content per dish accounts for both cellular and ECM proteins. Indeed, the mineralization potential significantly increased in D28 compared to D0 cultures, this increase being higher in the treated cells (Fig. 4C). Thus the lower cells sensitivity to Cd could be related to lower metal availability for uptake resulting from metal adsorption to the ECM. As depicted in Fig. 4D, ^{109}Cd adsorption to ECM estimated following hypotonic shock cell lysis increased as a function of days in culture possibly because of increased structure complexity of the produced ECM. This is further supported by the increased ^{109}Cd adsorption which is not inhibited (non-displaceable) by an excess of 100 μM unlabeled Cd. Note that at D0, ECM is relatively thin and flat and accumulate similar levels of Cd compared to Col I-coated dishes, suggesting the high Col I content in MC3T3 cells-secreted ECM.. Since Cd adsorption to ECM increases with cell differentiation, subsequent accumulation data were expressed per dish. As shown in Fig 4E, total accumulation of ^{109}Cd increased in D28 both untreated (D28B) and differentiated cells (D28T). Of interest, the non-displaceable (non-specific) component of ^{109}Cd accumulation increased with time in culture and with cell differentiation whereas the specific accumulation of ^{109}Cd remained unchanged (Fig. 4E, inset). Thus the increased nonspecific accumulation of Cd is likely mainly related to increased ECM secretion. In accordance with this conclusion, TRPM7 expression did not vary with time under either culture conditions (Fig. 4F). These results show that Cd uptake in MC3T3 is not modified by osteoblast differentiation whereas secreted bone-like matrix accounts for significant metal

adsorption. This may be in part responsible for the long biological half-life of 15-30 years estimated for Cd in humans (1,5).

Conclusion

Some studies stated that hypomagnesemia and hypocalcemia could be important risk factors in the development of osteoporosis (28). As demonstrated by their inhibitory effects, Ca and Mg supplementations could decrease long-term accumulation of Cd in osteoblasts. Moreover, our results show that Cd adsorption to cell-secreted ECM may protect osteoblasts from Cd cytotoxicity but also favour Cd storage in bone with potential risk when metal is released following bone resorption.

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References

(1) Huff, J., Lunn, R.M., Waalkes, M.P., Tomatis, L., and Infante, P.F. (2007). Cadmium-induced cancers in animals and in humans. *Int. J. Occup. Environ. Health* 13, 202-212.

(2) Staessen, J.A., Roels, H.A., Emelianov, D., Kuznetsova, T., Thijs, L., Vangronsveld, J., and Fagard,R. (1999). Environmental exposure to cadmium, forearm bone density, and risk of fractures: prospective population study. Public Health and Environmental Exposure to Cadmium (PheeCad) Study Group. *Lancet* 353, 1140-1144.

(3) Chalkley, S.R., Richmond, J., and Barltrop, D. (1998). Measurement of vitamin D_3 metabolites in smelter workers exposed to lead and cadmium. *Occup. Environ. Med.* 55, 446-452.

(4) Jarup, L. (2002). Cadmium overload and toxicity. *Nephrol. Dial. Transplant.* 17 Suppl 2, 35-39.

(5) Wilson, A.K., Cerny, E.A., Smith, B.D., Wagh, A., and Bhattacharyya, M.H. (1996). Effects of cadmium on osteoclast formation and activity in vitro. *Toxicol. Appl. Pharmacol.* 140, 451-460.

(6) Long, G.J. (1997). The effect of cadmium on cytosolic free calcium, protein kinase C, and collagen synthesis in rat osteosarcoma (ROS 17/2.8) cells. *Toxicol. Appl. Pharmacol.* 143, 189-195.

(7) Calvo, M.S., Eyre, D.R., and Gundberg, C.M. (1996). Molecular basis and clinical application of biological markers of bone turnover. *Endocr. Rev.* 17, 333-368.

(8) Manolagas, S.C. (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr. Rev.* 21, 115-137.

(9) Levesque, M., Martineau, C., Jumarie, C., and Moreau, R. (2008). Characterization of cadmium uptake and cytotoxicity in human osteoblast-like MG-63 cells. *Toxicol. Appl. Pharmacol.* 231, 308-317.

(10) Huang, C.L. (2004). The transient receptor potential superfamily of ion channels. *J Am. Soc. Nephrol.* 15, 1690-1699.

(11) Monteilh-Zoller, M.K., Hermosura, M.C., Nadler, M.J., Scharenberg, A.M., Penner, R., and Fleig, A. (2003). TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. *J. Gen. Physiol* 121, 49-60.

(12) Abed, E., Labelle, D., Martineau, C., Loghin, A., and Moreau, R. (2009). Expression of transient receptor potential (TRP) channels in human and murine osteoblast-like cells. *Mol. Membr. Biol.* 26, 146-158.

(13) Moreau, R., Aubin, R., Lapointe, J.Y., and Lajeunesse, D. (1997). Pharmacological and biochemical evidence for the regulation of osteocalcin secretion by potassium channels in human osteoblast-like MG-63 cells. *J. Bone Miner. Res.* 12, 1984-1992.

(14) Jumarie, C., Fortin, C., Houde, M., Campbell, P.G., and Denizeau, F. (2001). Cadmium uptake by Caco-2 cells: effects of Cd complexation by chloride, glutathione, and phytochelatins. *Toxicol. Appl. Pharmacol.* 170, 29-38.

(15) Shimoda, R., Nagamine, T., Takagi, H., Mori, M., and Waalkes, M.P. (2001). Induction of apoptosis in cells by cadmium: quantitative negative correlation

between basal or induced metallothionein concentration and apoptotic rate. *Toxicol. Sci.* 64, 208-215.

(16) Hinkle, P.M., Kinsella, P.A., and Osterhoudt, K.C. (1987). Cadmium uptake and toxicity via voltage-sensitive calcium channels. *J. Biol. Chem.* 262, 16333-16337.

(17) Pham, T.N., Segui, J.A., Fortin, C., Campbell, P.G., Denizeau, F., and Jumarie, C. (2004). Cadmium uptake in rat hepatocytes in relation to speciation and to complexation with metallothionein and albumin. *J. Cell Physiol* 201, 320-330.

(18) Bergeron, P.M. and Jumarie, C. (2006). Characterization of cadmium uptake in human intestinal crypt cells HIEC in relation to inorganic metal speciation. *Toxicology* 219, 156-166.

(19) Yamakage, M. and Namiki, A. (2002). Calcium channels--basic aspects of their structure, function and gene encoding; anesthetic action on the channels--a review. *Can. J Anaesth.* 49, 151-164.

(20) Clapham, D.E. (2003). TRP channels as cellular sensors. *Nature* 426, 517-524.

(21) Nadler, M.J., Hermosura, M.C., Inabe, K., Perraud, A.L., Zhu, Q., Stokes, A.J., Kurosaki, T., Kinet, J.P., Penner, R., Scharenberg, A.M., and Fleig, A. (2001). LTRPC7 is a Mg.ATP-regulated divalent cation channel required for cell viability. *Nature* 411, 590-595.

(22) Voets, T., Nilius, B., Hoefs, S., van der Kemp, A.W., Droogmans, G., Bindels, R.J., and Hoenderop, J.G. (2004). TRPM6 forms the Mg^{2+} influx channel involved in intestinal and renal Mg^{2+} absorption. *J Biol. Chem.* 279, 19-25.

(23) Abed, E. and Moreau, R. (2007). Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation. *Cell Prolif.* 40, 849-865.

(24) Bootman, M.D., Collins, T.J., MacKenzie, L., Roderick, H.L., Berridge, M.J., and Peppiatt, C.M. (2002). 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP3-induced Ca^{2+} release. *FASEB J.* 16, 1145-1150.

(25) Hu, H.Z., Gu, Q., Wang, C., Colton, C.K., Tang, J., Kinoshita-Kawada, M., Lee, L.Y., Wood, J.D., and Zhu, M.X. (2004). 2-aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. *J. Biol. Chem.* 279, 35741-35748.

(26) Yamauchi, M., Yamaguchi, T., Kaji, H., Sugimoto, T., and Chihara, K. (2005). Involvement of calcium-sensing receptor in osteoblastic differentiation of mouse MC3T3-E1 cells. *Am. J Physiol Endocrinol. Metab* 288, E608-E616.

(27) Jadlowiec, J., Koch, H., Zhang, X., Campbell, P.G., Seyedain, M., and Sfeir, C. (2004). Phosphophoryn regulates the gene expression and differentiation of NIH3T3, MC3T3-E1, and human mesenchymal stem cells via the integrin/MAPK signaling pathway. *J Biol. Chem.* 279, 53323-53330.

(28) Nieves, J.W. (2005). Osteoporosis: the role of micronutrients. *Am. J Clin. Nutr.* 81, 1232S-1239S.

Legends to the figures

Fig. 1 Cytotoxicity and uptake of Cd in MC3T3 cells. a) Time-course of cellular accumulation of 0.5 μM ^{109}Cd in the DME/F12 medium with 1mM Ca and 0.8 mM Mg. **(b)** Dose-response curve of cell viability (relative to control without Cd) following a 24-h exposure to increasing concentrations of Cd in DME/F12 with 1 mM Ca and 0.8 mM Mg. **(c)** 3-min uptake of 0.5 μM ^{109}Cd as a function of increasing concentrations of unlabeled Cd in the chloride transport medium.

Fig. 2 Effect of calcium and magnesium on Cd uptake and cytotoxicity. (a) Dose-response curves of cell viability (relative to control without Cd) following a 24-h exposure to increasing concentrations of Cd in control (Ctl) DME/F12 with 1mM Ca and 0.8 mM Mg (●) or in culture medium containing 0.1 mM Ca and 0.8 mM Mg (○) or 1 mM Ca without Mg (□). **(b)** 24-h uptake of 0.5 μM ^{109}Cd in DME/F12 with 1mM Ca and 0.8 mM Mg (Ctl), or in the presence of 5 mM Ca (+Ca) or 2.5 mM Mg (+Mg). **(c)** 3-min uptake of 0.5 μM ^{109}Cd in the Ca- and Mg-free nitrate medium (Ctl), or in the presence of 5 mM Ca (+Ca) or 2.5 mM Mg (+Mg). Significant difference compared to control (*$p<0.05$;**$p<0.01$, Dunnett). **(d)** 3-min uptake of 0.5 μM ^{109}Cd in the nitrate medium without Mg in the absence or presence of increasing concentrations of 2-APB. Significant difference compared to control (*$p<0.05$, Bonferoni).

Fig. 3 Effect of TRPM7 silencing on the Mg-sensitive component of Cd uptake. (a) mRNA analysis of TRPM7 channels relative to GAPDH following Mg starvation and siTRPM7 or siRNA(-) transfection. **(b)** 3-min uptake of ^{109}Cd in the absence (-Mg), presence (+Mg) of 2.5 mM Mg or following Mg-starvation for 24 h. Uptake was performed in the nitrate$^-$ medium in untransfected (Ctl), siTRPM7- or siRNA(-)-transfected cells. Significant difference (*$p<0.05$, Bonferoni) compared to the Mg-free condition. Significant differences (#$p<0.05$, ##$p<0.01$, Bonferoni) compared to the Mg-free Ctl conditions.

Fig. 4 Cd uptake and cytotoxicity as a function of osteoblast differentiation. (a) 28-days time-course of alkaline phosphatase (ALP) activity of untreated (basal) MC3T3 cells and cells cultured in differentiation medium (treated). Significant differences compared to the basal activity (*$p<0.05$, ***$p<0.0001$, Bonferoni). **(b)** LC_{50} values following a 24-h exposure to increasing concentrations of Cd as a function of time in culture in basal (○) or differentiation (●) media. Significant differences compared to basal conditions (*$p<0.01$, **$p<0.01$, Bonferoni). **(c)** Mineralization potential relative to control (D0) of MC3T3 cells following a 28-days additional culture period in the differentiation (treated) or basal medium. Significant difference compared to day 0 (***$p<0.0001$, Dunnett) or to untreated D28 cells ($^{\#\#\#}p<0.0005$, unpaired t test). **(d)** 3-min adsorption of 0.5 μM ^{109}Cd on Collagen type I-coated dishes or ECM at confluence (D0) or following a 28-days additional culture period in basal

(D28B) or differentiation (D28T) medium in the absence (total) or the presence of 100 μM unlabeled Cd (non displaceable). Significant difference (*$p<0.05$, **$p<0.01$, Bonferoni) compared to D0. **(e)** 3-min accumulation of 0.5 μM ^{109}Cd in the nitrate medium at confluence (D0) or following a 28-days additionnal culture period in basal (D28B) or differentiation (D28T) medium in the absence (total) or the presence of 100 μM unlabeled Cd (non-displaceable). Significant difference compared to D0 (*$p<0.05$, ***$p<0.001$, Dunnett). **(f)** mRNA analysis of TRPM7 channels relative to GAPDH at confluence (D0) or following a 28-days additional culture period in basal (D28B) or differentiation (D28T) medium.

Fig. 1 Cytotoxicity and uptake of Cd in MC3T3 cells

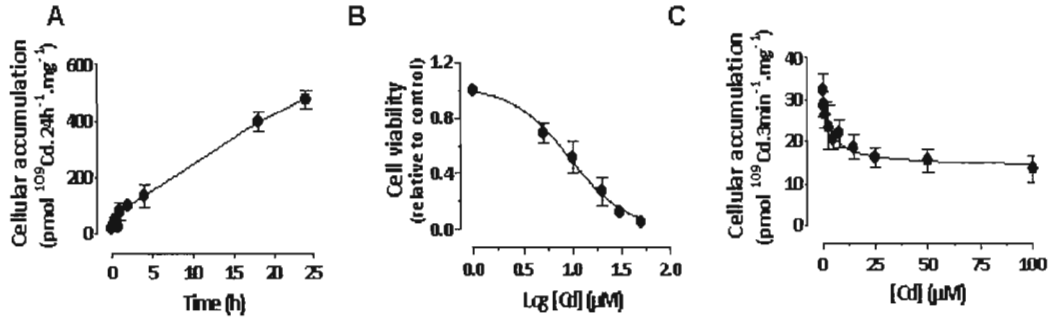


Fig. 2 Effect of calcium and magnesium on Cd uptake and cytotoxicity

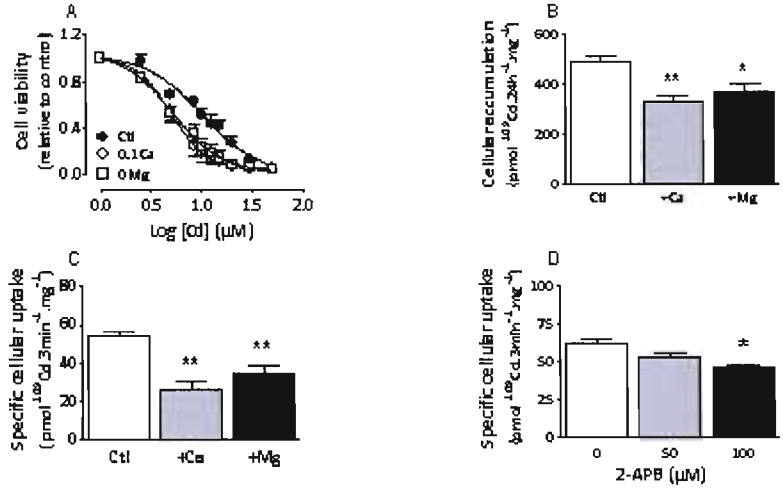


Fig. 3 Effect of TRPM7 silencing on the Mg-sensitive component of Cd uptake

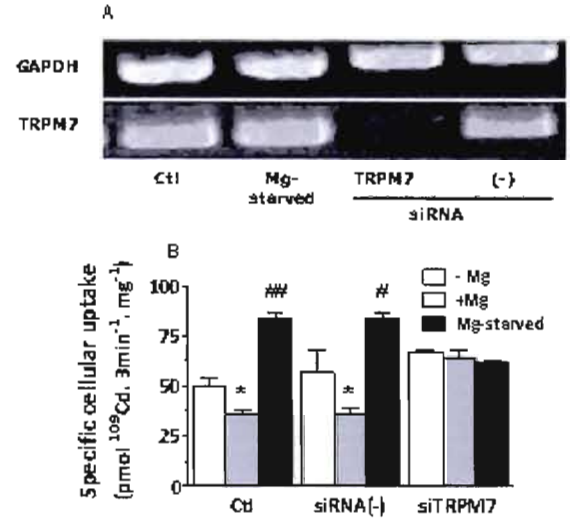
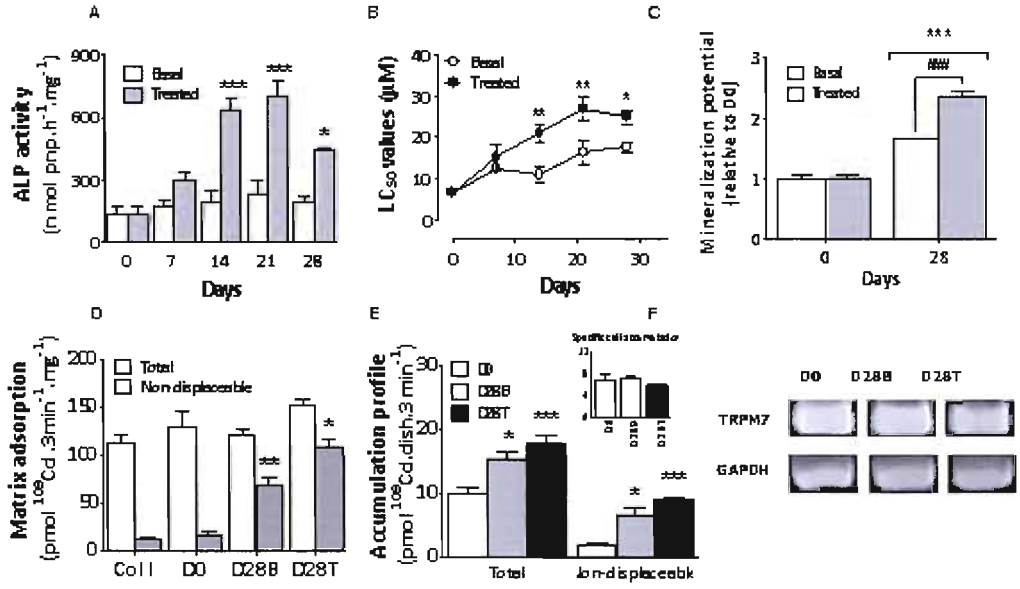


Fig. 4 Cd uptake and cytotoxicity as a function of osteoblast differentiation



APPENDICE C

Contributions avec comité de lecture

1) Abed É, Moreau R, (2008). Effet du magnésium et implication des canaux ''melastatin-related transient receptor potential 7'' (TRPM7) dans la prolifération et la migration des ostéoblastes induites par le « platelet-derived growth factor » (PDGF). 50 ieme réunion annuelle du C.R.C.Q. Les Presses de l'Université de Montréal, p121. Affiche retenue pour le concours Hans Selye. (Travaux de doctorat).

2) Abed É, Moreau R, (2008). Importance of Melastatin-like Transient Receptor Potential 7 and Magnesium in the Stimulation of Osteoblast Proliferation and Migration by Platelet-Derived Growth Factor The 30th ASBMR Annual Meeting, Montréal. J Bone Miner Res 23, S375. (Travaux de doctorat).

3) Abed É, Moreau R, (2007), L'expression des canaux ''melastatin-related transient receptor potential 7'' (TRPM7) dans la prolifération et la migration des cellules ostéoblastiques humains. 49 ieme réunion annuelle du C.R.C.Q. Med/Sci (v23, suppl.2, p6). Affiche retenue pour le concours Hans Selye. (Travaux de doctorat).

4) Abed É, Moreau R, (2007), Role of melastatin-like transient receptor potential 7 (TRPM7) channel in calcium influx and proliferation of human osteoblast-like cells. The 17th IBMS Scientific Meeting. Bone 40 (suppl.2, p S273). (Travaux de doctorat).

5) Abed É, Moreau R, (2006), Implication des canaux ioniques ''melastatin-related transient receptor potential 7'' (TRPM7) dans la prolifération des cellules ostéoblastiques. 48 ieme réunion annuelle du C.R.C.Q. Med/Sci (v2,

suppl.2, p6). Affiche retenue pour le concours Hans Selye. (Travaux de doctorat).

6) Abed É, Moreau R, (2005), Expression des canaux ''melastatin-related transient receptor potential 7'' (TRPM7) dans la prolifération des cellules ostéoblastiques. 47 ieme réunion annuelle du C.R.C.Q. Med/Sci (v21, suppl.2, p8). (Travaux de Maîtrise).

7) Martineau C, **Abed É,** Labelle D, Jumarie C, Moreau R. (2005), Cytotoxicité set voies d'entrée du cadmium dans les cellules ostéoblastiques. 47 ieme réunion annuelle du C.R.C.Q. Med/Sci (v21, suppl.2, p6). Affiche retenue pour le concours Hans Selye.

Contributions sans comité de lecture :

1) Abed É, Moreau R, (2008). Implication des canaux ''melastatin-related transient receptor potential 7'' (TRPM7) dans les fonctions des cellules ostéoblastiques humaines. (Séminaire de recherche, UQÀM)

2) Abed É, Moreau R, (2008). Effet du magnésium et implication des canaux ''melastatin-related transient receptor potential 7'' (TRPM7) dans la prolifération et la migration des ostéoblastes induites par le « platelet-derived growth factor » (PDGF). Biomed UQÀM.

3) Abed É, Moreau R, (2007), Rôles des canaux ''melastatin-related transient receptor potential 7'' (TRPM7) dans la prolifération et la migration des cellules ostéoblastiques humaines. Biomed UQÀM.

4) Martineau C, Lévesque M, **Abed É,** Labelle D, Jumarie C, Moreau R, (2007), Cytotoxicité et accumulation du cadmium dans différentes lignées ostéblastiques à divers degrés de maturité. Biomed UQÀM.

5) Abed É, Moreau R, (2006), Effet du Magnésium et implication des canaux TRPM7 dans la prolifération des cellules ostéoblastiques. Biomed UQÀM.

6) Abed É, Moreau R, (2006), Implication des canaux TRPM7 dans la prolifération des cellules ostéoblastiques humaines MG-63. (Séminaire de recherche, UQÀM)